PHYTOCHEMICAL INVESTIGATION AND ANTICANCER POTENTIAL OF THE SELECTED MEDICINAL PLANTS OF SESHACHALAM HILLS, ANDHRA PRADESH

A THESIS SUBMITTED TO

DELHI PHARMACEUTICAL SCIENCES AND RESEARCH UNIVERSITY,

NEW DELHI

FOR THE AWARD OF A DEGREE OF

DOCTOR OF PHILOSOPHY

IN

PHARMACEUTICAL SCIENCES

SUBMITTED BY

GARLA VENKATESWARLU
17/Ph.D./DPSRU/2020
AICTE-QIP Scholar

UNDER GUIDANCE OF

Dr. RAMESH BODLA
Associate Professor
Department of Pharmaceutical Chemistry
DIPSAR, NEW DELHI

RESEARCH CENTRE

DELHI PHARMACEUTICAL SCIENCES AND RESEARCH UNIVERSITY, NEW DELHI
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2024

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2024

DELHI PHARMACEUTICAL SCIENCES AND RESEARCH UNIVERSITY, NEW DELHI

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CERTIFICATE

This is to certify that the research work embodied in the thesis entitled **"Phytochemical Investigation and Anticancer Potential of the Selected Medicinal Plants of Seshachalam Hills, Andhra Pradesh"** for the degree of **Doctor of Philosophy (Pharmaceutical Sciences)** in the specialization has been carried out by **Garla Venkateswarlu** during the academic years **January/2021 to December/2024** at **Delhi Institute of Pharmaceutical Sciences and Research, Delhi Pharmaceutical Sciences and Research University.**

The thesis fulfills all the requirements and guidelines laid down for the **Doctoral Degree** from the Delhi Pharmaceutical Sciences and Research University (DPSRU). The work is up to my/our satisfaction.

30/12/2024

Director
DIPSAR

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Place: New Delhi

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This work was carried out under my guidance/supervision.

20/12/24

Research Guide

Dr. Ramesh Bodla

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DECLARATION BY THE CANDIDATE

I hereby declare that the thesis entitled **"Phytochemical Investigation and Anticancer Potential of the Selected Medicinal Plants of Seshachalam Hills, Andhra Pradesh"** submitted by me to the Delhi Pharmaceutical Sciences and Research University and Delhi Institute of Pharmaceutical Sciences and Research for the degree of **Doctor of Philosophy (Pharmaceutical Sciences)** in the specialization is the original piece of work carried out by me under the supervision and guidance of **Dr. Ramesh Bodla.** I further declare that it has not been submitted either in part or to any other University or Institute for the award of any degree/diploma/certificate course or for securing academic credits.

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Garla Venkateswarlu

Research Student

Date: 26/12/2024

Place: New Delhi

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This is certify that **Mr. Garla Venkateswarlu** Son of **Shri. Garla Venkataswamy** after having cleared Pre-Ph.D. course work and examination is now duly registered as a Ph.D. student from 1st **January 2021**. at Delhi Pharmaceutical Science and Research University.

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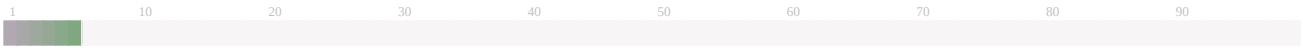

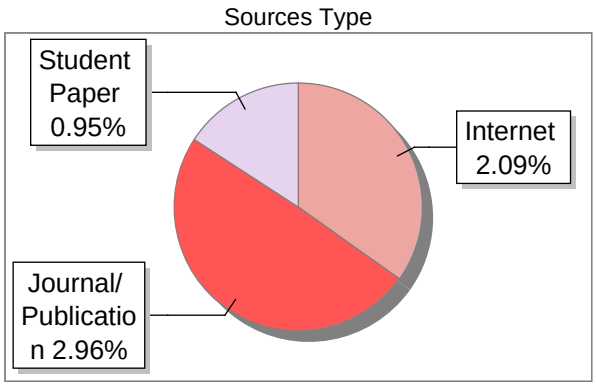

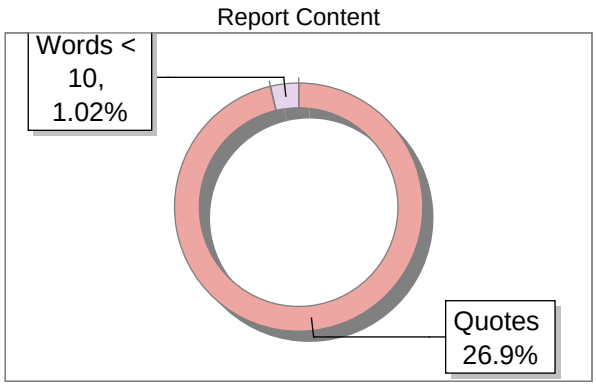

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Name of Supervisor: Dr. Ramesh Bodla
Date: 26/12/24

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ACKNOWLEDGEMENTS

I first owe my humble gratitude to the universe for bestowing on me the strength and courage to make my effort successful.

The financial support from the **All India Council for Technical Education (AICTE)** in the form of a **QIP (Quality Improvement Programme)** fellowship is gratefully acknowledged.

It was an immense pleasure for me to get an opportunity to pursue my Doctorate in **DIPSAR**, **DPSRU**, under **AICTE-QIP (Pharmacy)**, New Delhi.

I express my deep sense of gratitude and indebtedness to my esteemed Supervisor, Mentor **Dr. Ramesh Bodla,** Department of Pharmaceutical Chemistry, DIPSAR constituent college of DPSRU. I am very grateful to him for his valuable guidance and ever-lasting encouragement throughout my course.

I am immensely thankful to **Prof. Ravichandiran V**, Vice-chancellor, DPSRU and **Prof. P.K. Sahoo**, Offg. Registrar (DPSRU) & Director (DIPSAR) for providing the excellent facilities required for carrying out my research work.

The insights and expertise shared by **Prof. SV Suresh Kumar,** Professor of Pharmacognosy, Santhiram College of College of Pharmacy, Nandyala, Andhra Pradesh, have greatly enhanced my understanding of study, and their encouragement during difficult times has motivated me to persevere and achieve my goals.

I place regard my gratitude and immense respect on my esteemed Research Trainee Supervisor, **Dr. Shashank Kr. Singh**, Senior Principal Scientist, Pharmacology Division, CSIR – Indian Institute of Integrative Medicine, Canal Road, Jammu – 180001, for his affectionate encouragement, inspiring advice, and moral support in carrying out this dissertation work.

I owe a deep sense of gratitude to **Dr. Yedukondalu Nalli**, Scientist, Natural Products and Medicinal Chemistry Division, CSIR – Indian Institute of Integrative Medicine, Canal Road, Jammu – 180001, without his kind cooperation, deep faith and dynamic assistance the present work would not have seen the light of this day.

I am profoundly grateful to **Dr. Deepika Singh,** Principal Scientist, Quality Management & Instrumentation Division, CSIR – Indian Institute of Integrative Medicine, Canal Road, Jammu – 180001, for her time, constructive feedback, and commitment to my academic growth. Her expertise and perspectives have greatly enriched this work.

I extend my sincere gratefulness to the members of my Doctoral Student Research Committee, **Dr. Abdul Faruk**, Professor & Head, Department of Pharmaceutical Sciences HNB Garhwal University (A Central University), Uttarakhand-249161, **Dr. Jitendra Kumar**

Badjatya, Senior Manager RA, Emcure Pharmaceuticals Limited, Gandhinagar, Gujarat, India. Their expertise, constructive criticism, and valuable suggestions have significantly enhanced the quality of this research. I am grateful for their time, expertise, and commitment to fostering intellectual growth.

I am indebted to the faculty, Labmates and staff at **DIPSAR, DPSRU**, New Delhi, **S.V University**, Tirupati, Andhra Pradesh, **CSIR-IIIM**, Jammu for creating an environment conducive to research and learning. Their commitment to excellence and their tireless efforts to provide resources and support have been instrumental in my academic development.

I am deeply grateful to my family for their unwavering love, support, and encouragement. Their belief in my abilities and their constant motivation have been my anchor throughout this challenging journey. I am forever grateful for their sacrifices and understanding. This thesis is dedicated to my **ChinnaNanna, Shri Late Garla Venkata Ramanaiah**, whose unwavering love and support have been my foundation.

I would like to acknowledge to my guruji M Vijaya Nirmala Madam garu, my friends and colleagues who have stood by me and provided moral support during moments of doubt and frustration. Their camaraderie and shared experiences have been invaluable in maintaining my enthusiasm and resilience.

Lastly, I want to express my gratitude to all the teachers, mentors, and colleagues who have influenced and inspired me throughout my academic career. Their guidance, intellectual stimulation, and collaborative spirit have shaped my research trajectory and contributed to the person I am today.

GARLA VENKATESWARLU
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ABBREVIATIONS AND SYMBOLS

%	:	Per cent
ºC	:	Degree celsius
µ	:	Micron
µl	:	Microlitre
BO	:	*Boswellia ovalifoliolata*
H	:	Hexane
HA	:	Hydroalcohol
^{1}H-NMR	:	Proton nuclear magnetic resonance
^{13}C-NMR	:	Carbon-13 nuclear magnetic resonance
CC	:	Column chromatography
CD_3OD	:	Deutriated methanol
$CDCl_3$	:	Deutriated chloroform
CGM	:	Complete growth medium
$CHCl_3$	:	Chloroform
Conc.	:	Concentration
DAPI	:	4,6- diamidino-2-phenylindole
DCFDA	:	2,7-dichlorofluoroscein diacetate
DCM	:	Dichloromethane
DMSO	:	Dimethyl sulfoxide
DW	:	Distilled water
EDTA	:	Ethylene diamine-tetra acetic acid
ESIMS	:	Electrospray ionization mass spectrometry
et. al	:	et alia = and other
EtOH	:	Ethanol
FBS	:	Fetal bovine serum
FRAP	:	Ferric ion reducing antioxidant potential
g	:	Gram
GC-MS	:	Gas Chromatography-Mass spectroscopy
GI	:	Growth Inhibition
hr	:	Hour
HPLC	:	High-performance liquid chromatography
HPTLC	:	High-performance Thin Layer chromatography

IC_{50}	:	Inhibition concentration at 50
L	:	Litre
M	:	Molar
MeOH	:	Methanol
mg	:	Milligram
MHz	:	Mega hertz
MIC	:	Minimum inhibitory concentration
MMP	:	Mitochondrial membrane potential
min	:	Minutes
ml	:	Millilitre
mM	:	Millimolar
NMR	:	Nuclear magnetic resonance
OD	:	Optical density
ppm	:	Parts-per-million
ROS	:	Reactive oxygen species
rpm	:	Revolution per minute
RPMI	:	Roswell Park Memorial Institute Medium
SC	:	*Strychnos colubrina*
SRB	:	Sulphorhodamine blue
T	:	Toulene
TBST	:	Tris-buffered saline and tween-20
TCA	:	Trichloroacetic acid
TLC	:	Thin layer chromatography
VL	:	*Vitex leucoxylon*
v/v	:	Volume/volume
w/v	:	Weight/volume
µg	:	Microgram
WHO	:	World Health Organization

EXECUTIVE SUMMARY

Name of Research Scholar: Garla Venkateswarlu **Name of Supervisor:** Dr. Ramesh Bodla

Registration No. 17/Ph.D./DPSRU/2020

Title of thesis: Phytochemical Investigation and Anticancer Potential of the Selected Medicinal Plants of Seshachalam Hills, Andhra Pradesh

Abstract:

The research work is focused on the expedition of phytochemical and anticancer potential of medicinal plants - *Vitex leucoxylon*, *Strychnos colubrina*, and *Boswellia ovalifoliolata* - from Seshachalam Hills, Andhra Pradesh. The leaves of prioritized plants were collected, and extracts were prepared by sequential Soxhlet extraction method. Preliminary phytochemical analysis was carried out by standard phytochemical tests which revealed alkaloids, flavonoids, tannins, phenols, steroids, triterpenoids, anthocyanins, anthraquinones, and glycosides in the leaves of the selected plants. GC-MS analysis was revealed the presence of significant bioactive compounds. Isolation of compounds was monitored by TLC, HPTLC and confirmed by HPLC analysis, later characterized by IR, MS, HRMS, and NMR. SRB assay against a panel of three human cancer cell lines MCF-7 and HCT-116 disclosed the anti-cancer potentials of extracts with percent growth inhibition values at 10 µg/mL ranging from 70% to 90%. The anticancer constituents in the extracts were unfolded through a bio-assay-guided isolation process. Inugalactolipid-A, Cirsimarin, and Myricetin-7-Glucoside respectively of *Vitex leucoxylon, Strychnos colubrina,* and *Boswellia ovalifoliolata* are Bio-active constituents that attenuated the growth of colon cancer cells HCT-116 with an IC_{50} value of 19µg/mL, 8 µg/mL, and 6 µg/mL respectively and shown significant effects in DAPI, MMP and ROS assay.

Keywords: *Vitex leucoxylon*, *Strychnos colubrina*, *Boswellia ovalifoliolata*, phytochemical investigation, anticancer potential.

Introduction:

Medicinal plants have always played a pivotal role as sources for drug lead compounds. **Cancer** is a disease of the second leading cause of death globally. Natural products, particularly those of plant origin, have been used for the treatment of cancers since ancient times.

Seshachalam Hills, Andhra Pradesh, is the first biosphere reserve harboring many endemic and rare plants not yet explored for their phytochemical and anticancer potentials.

Objectives:

- Selection, collection, and authentication of medicinal plants

- Extraction and phytochemical analysis
- Isolation and structural elucidation of bioactive molecules
- Anticancer potential of extracts and isolated molecules

Methodology:

Plant Material collection and authentication:

The leaves of *Vitex leucoxylon*, *Strychnos colubrina*, and *Boswellia ovalifoliolata* were collected from Seshachalam hills, Tirupati, Andhra Pradesh. The plant species were authenticated by the Botanical Survey of India (BSI), Deccan Regional Center, Hyderabad-500095, Telangana.

Extraction, isolation, and phytochemical screening:

The powdered leaves of the prioritized plants were sequentially extracted by soxhlet apparatus with hexane, toluene, chloroform, ethanol, and hydroalcoholic (1:1). The extracts were dried and desiccated for further phytochemical and anticancer screening. Standard techniques were used to identify phytochemical components such as alkaloids, flavonoids, tannins, phenols, steroids and triterpenoids, anthocyanins, glycosides, saponins, and anthraquinones in extracts. After that fractionated and isolated active (ethanol) extract of *Vitex leucoxylon*, and *Strychnos colubrina* through the column bedded with silica 100-200 into hexane, DCM, DCM: MeOH, MeOH, and *Boswellia ovalifoliolata* active (ethanol) extract was loaded into the column containing HP20 and fractionated into the water, water: methanol, and methanol fractions.

***In-vitro cytotoxicity* screening (SRB assay):**

Seeded the human cancer cells (MCF-7, A-549, HCT-116) (100µl/well) in 96 well cell culture plates and incubated for 24 hr at 5% CO_2, 90% RH & 37°C. Added the test material (100 µl/well) and incubated for 48 hr at 5% CO_2, 90% RH & 37°C. Stopped the cell growth by the addition of chilled 50% TCA (50 µl/well), kept at 4°C for 1 hr washed the plates 5 times with distilled water, allowed the plates to dry, and added SRB dye into plates (100 µl/well) later plates were placed at room temperature for 30 min. Washed the plate with 1% acetic acid allowed the plates to dry and finally added Tris-Hcl buffer (100 µl/well) to dissolve the dye. Read the plates on the ELISA reader at 540 nM.

DAPI study: Cell lines (HCT-116) were seeded (12000 cells/well) in a six-well palate. After 24 hrs treated with Inugalactolipid-A, Cirsimarin, and Myricetin-7-Glucoside compounds separately. Camptothecin was used as a positive control. After 48 hrs washed with PBS and fixed with 4% paraformaldehyde for 15 min and washed with PBS. The cells were permeabilized with 0.1% triton-X for 7 min and washed with PBS. DAPI dye was added to the plates and kept in an incubator for 30 minutes. Finally, the cells were washed with PBS and

the images were taken *via* a confocal microscope.

MMP study: Cell lines (HCT-116) were seeded (12000 cells/well) in a six-well plate after 24 hrs. treated with Inugalactolipid-A, Cirsimarin, and Myricetin-7-Glucoside compounds separately. Camptothecin was used as a positive control. After 48 hrs discard the media, wash with PBS, add the Rhodamine-123 dye, and keep in an incubator for 20 min. Wash with PBS gently and take the images with a confocal microscope.

ROS study: Cell lines (HCT-116) were seeded (12000 cells/well) in a six-well plate after 24 hrs. treated with Inugalactolipid-A, Cirsimarin, and Myricetin-7-Glucoside compounds separately. After 48 hrs add the H_2O_2 as a positive control for 15 mins, then discard the media, wash with PBS add the H_2DCFDA dye, and keep in an incubator for 30 mi. Wash with PBS gently and take the images with a confocal microscope.

Characterization:

All extracts of *Vitex leucoxylon, Strychnos colubrina,* and *Boswellia ovalifoliolata,* were characterized using Brucker FTIR. The thin-layer chromatography profile of all extracts was performed to know the number of components present in extracts.

The pure isolated compounds of Inugalactolipid-A, Cirsimarin, and Myricetin-7-Glucoside were characterized from *Vitex leucoxylon, Strychnos colubrina,* and *Boswellia ovalifoliolata* by MS and NMR.

Results:

Successive hot continuous soxhlet extraction:

The physical characteristics of the toluene, chloroform, and ethanol extracts of *Vitex leucoxylon* are green color, amorphous powder, and solid whereas hexane extract shows light green, sticky semisolid, and hydroalcohol has light black, nonsticky powder, shiny particles. Extracts of *Strychnos colubrina* are hexane extract, which is light yellow, sticky, and solid, toluene extract shows a light green solid, and chloroform extract is dark green semisolid, sticky, and ethanol extract is dark green, sticky, semisolid, and hydroalcohol extract has brick red color, nonsticky, solid, powder. Extracts of *Boswellia ovalifoliolata* hexane extracts show a green color sticky, solid, and toluene extracts have a black color sticky, semisolid, and chloroform extract shows a buff color, solid, amorphous powder, ethanol extract shows a brown color, solid, nonsticky, and hydroalcohol extract have a brown color, amorphous powder, solid.

Preliminary phytochemical screening:

The initial screening of *Vitex leucoxylon* and *Strychnos colubrina* shows the presence of alkaloids, flavonoids, tannins, phenols, steroids & triterpenoids, anthocyanins, glycosides, saponins, and anthraquinone in four extracts and in toluene extract shows the absence of all

except alkaloids, flavonoids. Hexane and chloroform extracts show the absence of phytoconstituents, toluene extract shows the presence of all except anthraquinones, and ethanol and hydroalcohol extracts show the presence of phytoconstituents except alkaloids and anthraquinones of *Boswellia ovalifoliolata*.

Characterization:

The FT-IR spectrum showed hydroxyl (-OH), carbonyl (-CO-), and carboxylic acid (-COOH) functional groups in the extracts.

For the first time, we isolated Inugalactolipid A, Cirsimarin, and Myricetin-7-Glucoside from *Vitex leucoxylon, Strychnos colubrina,* and *Boswellia ovalifoliolata* respectively.

Inugalactolipid A:^{1}H-NMR (400 MHz, CD_3OD) δ 5.33-5.12 (m, 7H), 4.77 (d, J = 3.6 Hz,1H), 4.34 (dd, J = 12.2,2.9 Hz, 1H), 4.15 (d, J = 6.8 Hz, 1H), 4.13 (dd, J = 12.2, 6.8 Hz, 1H), 3.84 (dd, J = 10.6, 5.6 Hz, 1H), 3.83-3.73 (m,4H), 3.69 (dd, J = 10.6, 3.6 Hz, 1H), 3.66-3.54 (m, 6H), 3.44-3.35 (m, 2H), 2.72 (t, J = 5.7 Hz, 4H), 2.26-2.19 (m,4H), 2.04-1.95 (m, 4H), 1.55-1.47 (m, 4H), 1.29-1.17 (m, 32H), 0.88 (t, J = 7.5 Hz, 3H), 0.80 (t, J = 7.0 Hz, 3H);

^{13}C-NMR (100 MHz, CD_3OD) δ 175.1, 174.7, 132.7, 131.1, 129.2 (2C), 128.9, 128.2, 105.3, 100.6, 74.7, 74.6, 72.6,72.4, 71.7, 71.5, 71.1, 70.2, 70.1, 68.7, 67.8, 64.0, 62.8, 35.1, 35.0, 33.1, 30.8 (7C), 30.7, 30.5 (2C), 30.4, 30.3,30.2 (2C), 28.2, 26.6, 26.4, 26.1, 26.0, 23.8, 21.5, 14.7, 14.5.

Cirsimarin: ^{1}H NMR (400 MHz, Pyr) δ 7.96-7.82 (m, 2H), 6.93 (s, 1H), 6.80 (s, 1H),5.78 (d, J= 6.8 Hz, 3H), 4.63 (dd, J= 12.1, 2.1 Hz, 1H), 4.51-4.29 (m, 4H), 4.29-4.16 (m, 1H), 3.99 (s, 3H), 3.91 (s, 3H); ^{13}C NMR (100 MHz, Pyr) δ 183.35, 164.28, 161.63, 159.71, 153.82, 153.71, 133.38, 128.77, 125.13, 117.45, 106.63, 104.85, 101.90, 91.89, 80.03, 79.48, 78.76, 75.11, 71.54, 62.65, 60.83, 56.72.

Myricetin-7-Glucoside:^{1}H NMR (400 MHz, MeOD) δ 7.20 (s, 2H), 6.28 (d, J = 2.1 Hz, 1H), 6.10 (d, J = 2.1 Hz, 1H), 5.15 (d, J = 7.6 Hz, 1H), 3.63 (dd, J = 11.9, 2.4 Hz, 1H), 3.55-3.49 (m, 1H), 3.42 (dd, J = 8.8, 7.7 Hz, 2H), 3.35 (t, J = 8.8 Hz, 1H), 3.32 – 3.26 (m, 1H), 3.14 (ddd, J = 9.4, 5.1, 2.3 Hz, 1H); ^{13}C NMR (101 MHz, MeOD) δ 178.03, 164.62, 161.61, 157.61, 157.00, 145.02, 136.67, 134.40, 120.51, 108.58, 104.24, 103.06, 98.47, 93.25, 76.96, 76.78,74.30, 69.65, 61.02.

***In-vitro* anticancer study:**

In-vitro, cytotoxic evaluation disclosed growth inhibitory potential against a panel of three human cancer cell lines (MCF-7, A549, and HCT-116) with percent growth inhibition values ranging from 70-90% on HCT-116 at 20 μg/mL. The active extracts were further fractionated and subjected to *in vitro* cytotoxicity screening against the HCT-116 human cancer cell line.

The IC_{50} values were also evaluated for the active fraction. The IC_{50}values were in the range of 11 to 40 µg/mL with Fraction 8 of *Vitex leucoxylon* showing the lowest IC_{50} value of 19µg/mL against HCT-116 and Fraction 5 of *Strychnos colubrina* showing an IC_{50} value of 5 µg/mL against HCT-116. The active fractions on sub-fractionation led to the isolation of Inugalactolipid-A, Cirsimarin, and Myricetin-Glucoside respectively from *Vitex leucoxylon, Strychnos colubrina* and *Boswellia ovalifoliolata* for the first time. Inugalactolipid-A, Cirsimarin, and Myricetin-7-Glucoside showed an IC_{50} value of 19, 8, and 6 µg/mL against HCT-116.

Shown significant activity in DAPI, MMP, and ROS studies at 20 µg/mL than all concentration.

Conclusions:

The results confirmed that *Vitex leucoxylon*, *Strychnos colubrina;* and *Boswellia ovalifoliolata* contained several phytoconstituents such as alkaloids, flavonoids, tannins, phenols, steroids & triterpenoids, anthocyanins, anthraquinones, and glycosides that may contribute to the anticancer potential of *Vitex leucoxylon*, *Strychnos colubrina,* and *Boswellia* ovalifoliolata. The active compounds were isolated and screened for their anticancer potential. Detailed biological studies will provide the compound(s) therapeutic potential.

Outcomes of the overall work done:

- The three novel metabolites of Inugalactolipid-A, Cirsimarin, and Myricetin-7-Glucoside were isolated from the selected medicinal plants.
- Three isolated phytoconstituents showed significant anticancer potential against colorectal (HCT-116) cancer.

Signature of Student **Signature of Supervisor**

LIST OF PUBLICATIONS

- Garla Venkateswarlu, Shashank Kr Singh, Ramesh Bodla. Phytochemical Evaluation and GC-MS Analysis of Ethanolic Extract of Endemic Plant of Boswellia ovalifoliolata Leaves, Journal of Natural Remedies, Volume 25, Issue 1 (Accepted).
- Garla Venkateswarlu, Ramesh Bodla. Phytochemical Investigation for Secondary Metabolites of Selected Endemic Medicinal Plants of Seshachalam hills, Andhra Pradesh, Biochemical and Cellular Archives, Vol. 24, September 1, 2024.
- Venkateswarlu G, Bodla R. Phytochemical Evaluation and Gc-Ms Analysis of Ethanolic Leaves Extract of Vitex Leucoxylon, Afr.J.Bio.Sc. 6(10), (2024), 5353-5368.
- Garla Venkateswarlu, Ramesh Bodla, Phytochemical Evaluation and Gc-Ms Analysis of Ethanolic Leaves Extract of Strychnos Colubrina, Afr.J.Bio.Sc. 6(5), (2024). 5871-5887.
- Garla Venkateswarlu, Sachin Kumar, Bodla R. Examining The Utility of Models in Evaluating Anticancer Therapies: From Lab to Clinic. Acta Biomed 2023; Vol. 94, N. 1: ISSN: 0392-4203 | eISSN: 2531-6745.
- Bukke SP, Gali AK, Igbinoba SI, Garla V, Hussaini B, Goruntla N, Onohuean H. Anti-Apoptotic and Anti-Inflammatory Protective Mechanisms of Gmelina Arborea Stem Bark Extract on Ischemic Reperfusion Injury in Albino Wistar Rats. RPS Pharmacy and Pharmacology Reports. 2024 May 31:rqae015.
- Suchitra M, Kumar KP, Katagi MS, Venkateswarlu G, Mahalakshmi PS. HDAC Inhibitors: A Novel Approach to Hyperglycaemia Management and Treatment. Health Sciences Review. 2023 Nov 14:100137.

BOOK CHAPTERS:

- Garla Venkateswarlu, Sachin Kumar, Suddikattu Bhargavi, Ramesh Bodla, Chapter-2: The Biology of Cancer: Understanding the Disease and its Challenges, Biosensors and Aptamers: A New Era in Cancer Diagnosis and Treatment, Springer Nature, 2024.
- Molakpogu Ravindra Babu, Shaik Abdul Rahaman, Garla Venkateswarlu, Yachana Mishra, Vijay Mishra, Chapter-2, Nanomedicine Research, Development, and Current Clinical status, Intelligent Nanobiosystems in Medicine and Healthcare, Volume 1, Elsevier Publications, 2024.

PATENT:

- Garla Venkateswarlu, Ramesh Bodla, Shashank Kr Singh, Deepika Singh, Vandana, Yedukondalu Nalli “Isolation of cirsimarin from *Strychnos colubrina*” (process patent

under communication by CSIR-IIIM, Jammu), 2024.

CONFERENCE PRESENTATIONS:

- Poster presented on "Phytochemical evaluation, TLC profiling, and FT-IR analysis of *Vitex leucoxylon* leaves" at "One Week One Lab" held on June 4th-10th 2023, at CSIR-Indian Institute of Integrative Medicine (CSIR-IIIM), Jammu, India.
- E-poster presented on "Phytochemical investigation and FT-IR analysis of the selected medicinal plants of Seshachalam hill ranges, Andhra Pradesh" at 5th International Pharma Conference (5th Pharma-2023 Online) held on May 23-24, 2023, in Hyderabad, India.
- Presented oral poster and got 1st prize on "Phytochemical screening of the selected medicinal plants of Seshachalam hills, Andhra Pradesh" at PHARMA ORANE 2K23 NATIONAL CONFERENCE on 31st March-1st April 2023, M.A.M College of Pharmacy, Narasaraopet, Palnadu, Andhra Pradesh, India.
- Participated in 4th Annual Conference of Society for Alternatives to Animal Experiments- India (SAAE-I) on 11th and 12th December, 2021, In association with Delhi Pharmaceutical Sciences and Research University, New Delhi.
- Poster presented on "Plant Secondary Metabolites as an Anti-cancer agent" at 18th INDO-US INTERNATIONAL CONFERENCE on 11th day of June 2022, M.A.M College of Pharmacy, Narasaraopet, Guntur, India.
- Presented as a co-author on "Microbial Biotransformation: An Effective Alternative to The Conventional Synthesis of Compounds (G-55)" at 72th Indian Pharmaceutical Congress on January 20-22, 2023, in Nagpur, India.
- E-poster presentation on "Pharmaceutical agents as Anti-cancer agents" at 3rd International Pharma Conference (3rd Pharma-2021 Online) on December 17-18, 2021, in Hyderabad, India.

FACULTY DEVELOPMENT PROGRAM:

- Participated in National e-FDP on "FOSTERING THE DRUG DESIGN VIA ARTIFICIAL INTELLIGENCE AND SANCTIFYING MOLECULAR DOCKING STUDIES" from 24.07.2023–28.07.2023, organized by the Department of Pharmaceutical Chemistry and Analysis, School of Pharmaceutical Sciences, VISTAS, Chennai, India.

CONTENTS

S. No.	Particulars	Page No.
	Certificates	
	Acknowledgements	
	List of Tables	**(i-ii)**
	List of Figures	**(iii-viii)**
	Abbreviations & Symbols	**(ix-x)**
	Executive summary	**(xi-xv)**
	Publications	**(vii)**
CHAPTER 1:	**INTRODUCTION**	**1-6**
CHAPTER 2:	**OBJECTIVES`**	**7**
CHAPTER 3:	**REVIEW OF LITERATURE**	**8-16**
CHAPTER 4:	**MATERIAL AND METHODS**	**17-32**
CHAPTER 5:	**RESULTS AND DISCUSSION**	**33-83**
CHAPTER 6:	**SUMMARY & CONCLUSIONS**	**84-85**
CHAPTER 7:	**REFERENCES**	**86**

Phytochemical Investigation and Anticancer Potential of the Selected Medicinal Plants of Seshachalam Hills, Andhra Pradesh

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Registration No. 17/Ph.D./DPSRU/2020

1. INTRODUCTION:

Plants have been the cornerstone of traditional medicinal practices worldwide and continue to provide humanity with innovative remedies. The World Health Organization (WHO) describes traditional medicine, with herbal remedies, as therapeutic practices that have existed for centuries, predating current medicine, and remain in use today. Herbal medicine is the synthesis of the therapeutic experience of a generation of working physicians of ethnic systems of medicine (Jamshidi-Kia, F., *et al*., 2017).

Herbal medicines are utilized for their safety, efficacy, cultural acceptability, and reduced adverse effects. The chemical elements found in plants contribute to the physiological activities of living systems, so they are considered to exhibit greater compatibility with the human body. These pharmaceuticals are derived from renewable raw materials through environmentally sustainable techniques and will foster economic development (Welz, A. N., *et al*., 2019).

India is unique of the globe 12th biodiversity zones by the existence of over 45 thousand diverse herbal species, though only half of the plants are used for their medicinal values. Ayurveda, Siddha, Unani, and Folk medicines are the indigenous medicinal systems in India. About 8,000 herbal medicines consume in Ayurveda. The Rigveda enumerates 67 medicinal plants, the Yajurveda lists 81 species, the Atharvaveda identifies 290 species, while the Charak Samhita and Sushruta Samhita examine the qualities and applications of 1100 and 1270 species, respectively, which continue to be utilized in traditional formulations (Seth, S. D., & Sharma, B. 2004).

Medicinal plants are pivotal in the development of powerful therapeutic agents, with plant-based drugs making significant contributions to modern medicine. Plant based drugs are used to cure, cancer, skin diseases, tuberculosis, hypertension, mental illness, diabetes, and jaundice. The utilization of botanical substances as indigenous remedies in folklore or traditional medicinal practices.

Listed the few traditionally used medicinal plants for the treatment of different diseases in Table.1.

Table.1. List of few traditionally used medicinal plants for the treatment of different diseases

S. No	Name of the Medicinal Plants	Used in the disorder
1	*Withania somnifera, Terminallia arjuna, T. chebula, Cyperus rotundus, Phyllanthus embellica, Osmium sanctum*	Heart tonic
2	*Cyperus rotundus, Tinospora cordifolia, Azadiracta indica, Terminallia chebula, Phyllanthus embellica, Curcuma longa, Centella asiatica*	Skin disease
3	*Crateva nurvala, Sida cordifolia, Terminallia chebula, Glycyrrhiza glabra, Zingiber officinalis*	Thyroid problems
4	*Holarrhena antidysenterica, Phyllanthus embellica, Zingiber officinalis, Terminallia chebula, Cyperus rotundus*	Diarrhoea
5	*Solanum xanthocarpum, Piper longum, Adhatoda vasaka, Zingiber officinalis, Phyllanthus embellica, Osmium sanctum*	Asthma
6	*Azadiracta indica, Terminallia chebula, Holarrhena antidysenterica, Tinospora cordifolia, Vinca rosea, Bauhinia variegate, Crateva nurvala*	Cancer

Andhra Pradesh is a state located in the southeastern coastal region of India. It is the seventh-largest state by area, covering 162,975 km² (62,925 sq mi), and the tenth-most-populous state, with a population of 49,386,799" (Figure No.1.). It is also a major producer of rice known as the "Rice bowl of India". The state of Andhra Pradesh boasts a diverse topography, ranging from the hills of the Eastern Ghats and Nallamala Hills to the shores of the Bay of Bengal. This variety supports a rich diversity of ecosystems, as well as a wide range of flora and fauna (Reddy, C. S., 2009). "The plains to the east of the Eastern Ghats form the Eastern Coastal plains". The Eastern Ghats in Andhra Pradesh are uneven, with separate slices having local names. They act as a major geographic dividing line in the state. The region is categorized by dense tropical forests, which become sparser as the Ghats transition into the Deccan Plateau, where shrub vegetation is more prevalent. The state's vegetation consists largely of dry deciduous types, including species like teak, *Terminalia*, *Dalbergia*, *Pterocarpus*, and *Anogeissus*. Andhra Pradesh is home to some erratic and endemic plants, such as *Cycas*

beddomei, Pterocarpus santalinus, Psilotum nudum Terminalia pallida, Shorea talura, Shorea tumburgia, and *Syzygium alternifolium*. Tirupati, commonly referred to as the "Spiritual Capital of Andhra Pradesh" (Guptha, M. B., *et.al*, 2012).

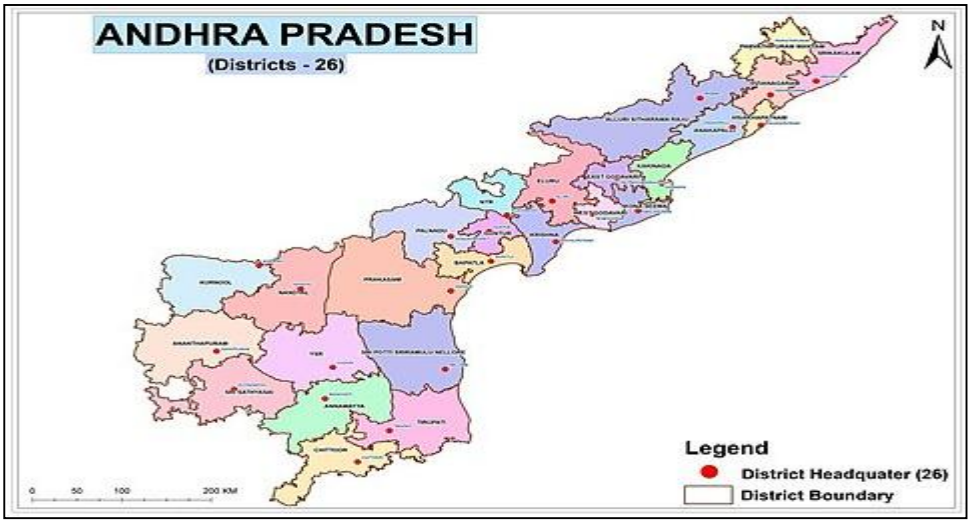


Figure No.1. Map of Andhra Pradesh

The Eastern Ghats possess significant floristic variety and harbor several uncommon, endemic, and endangered plants. Vegetation exhibits significant variation with height, displaying a clear zonation of forest types, including scrub jungles, deciduous woods, dry and semi-evergreen forests, evergreen forests, and sholas. This region is home to around 4,500 species of Angiosperms, representing approximately 13% of India's blooming plants.

The Seshachalam hill ranges were formed during the Precambrian supereon, spanning from 3.8 billion to 539 million years ago (Figure No.2.). These hills contain minerals such as sandstone and shale, interbedded with limestone (Figure No.3). They are bounded by the Rayalaseema uplands to the west and northwest, and the Nandyal Valley to the north" (Reddy, A. M., *et.al*, 2019).

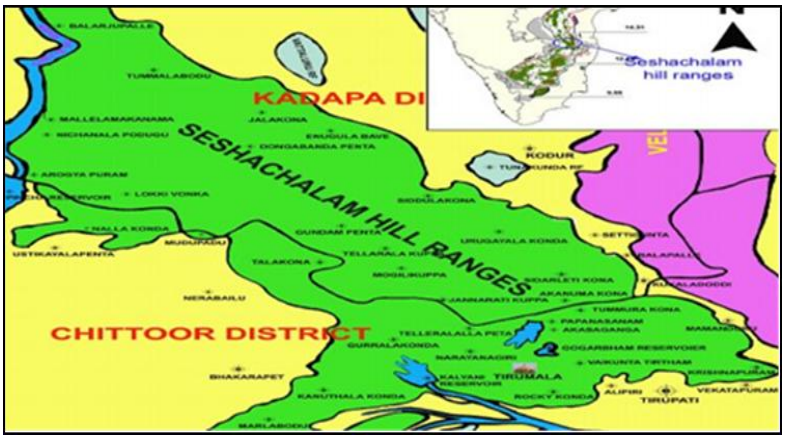


Figure No.2. Map of Eastern Ghats of Seshachalam hill ranges, Tirupati

Cancer is a multifaceted group of diseases that can rise in nearly any tissue in the body. A complete cancer is characterized by cells that have develop categorically antisocial and carry on their actions irrespective of the surrounding cells and tissues. Normal cells have an uncountable of mechanisms that monitor their occupied in cooperation with other cells. Cancer cells disrupt this normal activity, they divide at their will regardless of how much they crowd their neighbors, move to places they are not belonging to, attract blood vessels to themselves, and stop obeying aging signals. It has therefore been stated that cancer cells behave badly and their damage gives rise to tumors. For each cancer has its specific exclusive arrangement of activities which is determined by the tissue in which it is formed, the mutations cells have espoused and the interaction in a person's body. The development of cancer (carcinogenesis) is a multistage process and is initiated by an external agent i.e. a carcinogen e.g. a virus, an exogenous chemical compound, or ionizing radiation. Normally after entering the body these carcinogens are either excreted (chemicals), deactivated (chemicals or radiation), or inhibited (viruses), however, if any of the carcinogens are not neutralized by one of these mechanisms, it activates a normal cell and eventually induces a genetic mutation (Weinberg, R. A., & Weinberg, R. A. 2006).

The World Health Organization (WHO) states that 80% of those residing in rural regions depend on medicinal plants as their principal healthcare system. Nowadays, most research on cancer drugs is targeted at plants and their derived natural products. Plant-derived substances have been a crucial source of numerous clinically effective anticancer medicines. Included are vinblastine, paclitaxel, vincristine, camptothecin compounds such as topotecan and irinotecan, etoposide from epipodophyllotoxin, homoharringtonine, and elliptinium (Greenwell, M.,. *et al*., 2015) The isolation of the vinca alkaloids vincristine and vinblastine from the Madagascar periwinkle, *Catharanthus roseus*, marked the beginning of a new era in the use of plant-derived materials as anticancer agents. They were the first plant-derived agents used for cancer treatments. The discovery of paclitaxel, a drug extracted from the bark of the Pacific Yew tree (*Taxus brevifolia*), highlights the success of natural product drug discovery. Paclitaxel is widely used in the treatment of lung cancer, advanced breast cancer, ovarian cancer, both small and non-small cell cancer (Jain, S., *et al*., 2016). Additionally, in traditional Indian Ayurvedic medicine, the related *Taxus baccata* tree was used to treat cancer, demonstrating its historical relevance in cancer treatment. Another important discovery is camptothecin, isolated from the Chinese ornamental tree *Camptotheca acuminata*. This compound was advanced to clinical trials by the National Cancer Institute (NCI) in 1970. Camptothecin led to the development of two semi-synthetic derivatives: topotecan and irinotecan. These drugs are used to treat a range

of cancers, including ovarian cancer, small cell lung cancer, and colorectal cancer, showing how natural compounds can be modified to create effective treatments for various types of cancer.

Epipodophyllotoxin, an active anti-tumor agent, is isolated from the roots of plants in the Podophyllum species and has shown significant anticancer activity against a variety of cancers. Two semi-synthetic derivatives of epipodophyllotoxin, etoposide and teniposide, are commonly used in the treatment of testicular cancers and lymphomas.

When choosing a plant for pharmacological studies, four basic methods are commonly employed:

Random choice of plant species – Selecting plants without a specific guiding principle or criteria.

Choice based on ethnomedical use – Choosing plants that have been traditionally used in local medicine to treat certain ailments.

Follow-up on existing literature – Selecting plants that have already been researched or documented for their medicinal properties.

Chemotaxonomic approaches – Selecting plants based on their chemical characteristics and classification.

A comparison of these four methods has shown that the ethnomedical approach (based on traditional use) provides about 25% more positive leads for discovering pharmacologically active compounds compared to the other methods. In the present study, plant selection was based on the ethnomedical use and the existing literature on the species, leveraging traditional knowledge and previous research findings to guide the selection process (Samy, R. P., *et al*., 1999, Carlson, T. J., *et al*., 1997).

***In vitro* Cytotoxicity**

Some confusion exists in using the terms ‘Cytotoxicity’, ‘antineoplastic,’ and ‘antitumor.’ The National Cancer Institute (NCI) has defined these terms: Cytotoxicity refers to *in vitro* toxicity of tumor cells. At the same time, antineoplastic and antitumor should refer to *in vivo* activity in experimental systems. *Calophyllum inophyllum* Linn. is a tree that grows 8 to 20 meters tall and belongs to the family Clusiaceae. The dried leaf and its decoction cure rheumatism, skin infections, cuts, and sores (Jain, R., & Jain, S. K. 2011). The fresh leaves infusion is used to cure bacterial and fungal infections and as vermifuge/pediculicide. The (+) - calanolide A and inophyllum B isolated from Calophyllum lanigerum Miq. and Calophyllum inophyllum L. showed potent activity against Human Immuno Deficiency Virus type 1. *Caesalpinia pulcherrima L.,* commonly known as the Peacock-flower or Pride of Barbados, is a perennial

shrub or small tree that belongs to the Fabaceae family. Phytochemical screening on *Caesalpinia pulcherrima* have revealed the presence of various phytoactive components such as isoflavones, glycosides, rotenoids, chalcones, flavonols, flavanones, flavones, and sterols. Leaves have shown antitumor activity, while seeds have demonstrated antiviral activity. Its stem had shown cytotoxic activity (Srinivas, K. V. N. S., *et al*., 2003).

Hence, the present study is focused on examining the phytochemistry and anticancer activities of *Vitex leucoxylon*, *Strychnos colubrina*, and *Boswellia ovalifoliolata* leaves.

Rationale of the Study

The Seshachalam Hills, located in Andhra Pradesh, India, are part of the Eastern Ghats and are known for their rich biodiversity, including a wide variety of medicinal plants. Traditional medicine systems, such as Ayurveda, have long utilized these plants for treating various ailments. However, despite their extensive use in traditional practices, many of these plants remain underexplored in terms of their phytochemical constituents and therapeutic potential, particularly their anticancer properties (Ganesh, P., & Sudarsanam, G. 2013).

Cancer remains one of the leading causes of death worldwide, and the search for effective, safe, and affordable treatments is ongoing. Medicinal plants have gained significant attention as potential sources of novel anticancer agents due to their bioactive compounds, such as alkaloids, flavonoids, terpenoids, and phenolics, which have shown promising activity in inhibiting cancer cell proliferation and inducing apoptosis (Bray, F., *et al*., 2018, Ferlay, J., *et al*., 2021).

Given the ecological significance of the Seshachalam Hills and the potential of its flora, this study aims to conduct a comprehensive phytochemical investigation of selected medicinal plants from this region. The goal is to identify and isolate bioactive compounds with potential anticancer properties. By exploring the phytochemistry of these plants, the study seeks to contribute to the discovery of new anticancer agents, which could lead to the development of more effective and less toxic cancer treatments.

Moreover, this research will also help in the conservation of the medicinal plants of the Seshachalam Hills by highlighting their potential therapeutic value, thereby promoting sustainable use and preventing their overexploitation. The study's findings could pave the way for further pharmacological studies and the development of plant-based anticancer drugs, reinforcing the importance of preserving biodiversity for future scientific discoveries.

2. AIM & OBJECTIVES:

The present study aim is to investigates the phytochemical, and anticancer potential of the selected medicinal plants of Seshachalam hills, Andhra Pradesh.

In this background, the objectives of the present study are: -

A thorough literature survey was carried out for selected plants and anticancer activity before starting the experiment and has also been updated regularly.

- To select, collect and authenticate of three medicinal plants of *Vitex leucoxylon, Strychnos colubrina,* and *Boswellia ovalifoliolata* from Seshachalam hills, Andhra Pradesh.
- To extract and perform the phytochemical analysis of the crude extracts of three medicinal plants of *Vitex leucoxylon, Strychnos colubrina,* and *Boswellia ovalifoliolata*.
- To identify, isolate and characterize the active principles(s) from the active crude extract of *Vitex leucoxylon, Strychnos colubrina* and *Boswellia ovalifoliolata* responsible for an anticancer property using column chromatography, TLC, HPTLC, IR, GC-MS, MS, HRMS and NMR.
- To investigate the *in vitro* anticancer activity of leaf extracts and isolated molecules using Sulforhodamine B (SRB) assay on Michigan Cancer Foundation-7 (MCF-7), Lung carcinoma epithelial cells (A549), and Colorectal Carcinoma (HCT116) cell lines with further mechanistic studies like 4',6-diamidino-2-phenylindole **(**DAPI), Mitochondrial membrane potential (MMP), and Reactive oxygen species (ROS).

Statistical Analysis:

Data analysis was conducted with MS-Excel and GraphPad Prism 5 software. All data analysis was conducted with GraphPad Prism-5 Software and Image-J. The statistical significance of the data was assessed using one-way analysis of variance (ANOVA) and approved at $P<0.05$.

3.LITERATURE REVIEW:

Plant Species Used in Clinical Cancer Treatment

Nearly 15 species across ten genera and nine families have been utilized in clinical cancer chemotherapy. Notably, mainstream antitumor agents are derived from genera such as *Camptotheca*, *Podophyllum*, *Taxus*, and *Vinca*. The timeline of FDA-approved natural compound-based anticancer drugs from these species.

***Camptotheca acuminata*:**

A native tree of Southern China and Tibet, *Camptotheca* is protected but cultivable. Traditionally used to treat stomach, liver, and leukemia cancers, it contains the quinoline alkaloid camptothecin (CPT), which inhibits topoisomerase I and DNA replication. Due to CPT's hematological toxicity, semisynthetic derivatives like topotecan and irinotecan have been developed, offering reduced side effects (Wani, M. C., & Wall, M. E. 1969).

2. Podophyllum species:

Podophyllum peltatum, a North American herb, contains toxic podophyllotoxins that inhibit mitosis by blocking microtubule assembly. Derivatives such as etoposide and teniposide are effective against small-cell lung cancer, testicular carcinoma, and lymphoma. Increased podophyllotoxin production has also been observed in *P. hexandrum* and *P. emodi* (Chaurasia, O. P., *et al*., 2012).

3. Vinca rosea

Commonly known as Madagascar periwinkle, this tropical weed contains alkaloids like ajmalicine, vindoline, and catharanthine. These alkaloids form cytotoxic dimeric compounds, including vinblastine and vincristine, effective against leukemia, skin cancer, and Hodgkin's disease (Johnson, I. S., *et al*., 1960).

4. Mistletoe:

A semi-parasitic shrub, mistletoe contains viscotoxins and lectins that exert cytotoxic effects on tumor cells and stimulate immune responses. Despite its toxicity in high doses, mistletoe preparations are well-tolerated (Khwaja, T. A., *et al*., 1986).

5. Taxus species:

Taxus baccata (English yew) and *Taxus brevifolia* (Pacific yew) produce paclitaxel, a taxane diterpene that inhibits mitotic spindle breakdown, preventing cell division. Paclitaxel and its derivative docetaxel are effective against ovarian and advanced breast carcinomas (Hafezi, K., *et al*., 2020).

6. Chelidonium:

This urban weed contains chelidonine and its derivative Ukraine, which restores macrophage function. It has shown promise against Kaposi's sarcoma and astrocytoma (Wouters, A., *et al*., 2015).

7. Paeonia species:

Root preparations from *Paeonia alba*, *P. lactiflora*, and *P. suffruticosa* have been used to treat gastric mucosa abnormalities, including intestinal metaplasia.

8. Rabdosia rubescens:

A Chinese medicinal plant, *Rabdosia* contains oridonin, effective in treating esophageal carcinoma (Sartippour, M. R., *et al*., 2005).

9. Rubiacordifolia:

An Indian plant, *Rubia* contains anthraquinones, naphthoquinones, and hexapeptides (e.g., RA-XI to RA-VII) with antitumor activity. Despite promising results, some compounds have shown undesirable toxicity (Son, J. K., *et al*., 2008, Ghosh, S., *et al*., 2010).

3.1 PLANT PROFILE:

Plants have been a basis of medicine for decades and continue to provide a wealth of effective bioactive compounds for the treatment of diseases (Sao, A., *et al*., 2024) Medicinal plants possess bioactive secondary metabolites, which are accountable for their therapeutic activities. Medicinal plants with established healing powers function as healthcare entities (Bukke, S. P. N.,*et al*., 2024, Suchitra, M., *et al*., 2023). An urgent necessity is thus crucial for their accurate identification and application. The understanding of plant internal structure is crucial for addressing various prevalent issues, including the identification of mysterious specimens, food contamination, and forensic challenges (Gurib-Fakim, A. 2006). List of selected medicinal plants for this study was shown in table no.1.

Table No.1: List of selected medicinal plants

S. No	Plant Name	Family
1	*Vitex leucoxylon* L.f.	Lamiaceae
2	*Strychnos colubrina*	Loganiaceae
3	*Boswellia ovalifoliolata*	Burseraceae

1. *Vitex leucoxylon* L.f.

Vitex leucoxylon L.f. is an endemic tree found in peninsular India and Sri Lanka. It spreads a height of 12 meters. Leaves are 3- 7 foliolate with an elongate petiole of about 10 cm. Flowers are in corymbose panicles, cream and purplish within. Fruits are seen all over the year. Trees

are mostly seen along riverbanks and hills. *Vitex leucoxylon* (figure no. 8) is one of the useful medicinal trees in India. The pharmaceutically active extracts of *V. leucoxylon* have shown hypoglycemic and anti-inflammatory properties. Stem decoction to cure whooping cough, dried leaf powder smoke as a remedy for asthma, leaf paste to wash scars of pox wounds, root bark to reduce fever, fresh twigs as insecticides, and dry leaf powder to preserve food grains. Leaf extract is also suggested by native healers for inhibiting the increase of blood sugar. It is used as a traditional medicine for relieving headaches and catarrh. The pharmacological studies revealed anti-psychotic, anti-depressant, analgesic, anti-inflammatory, anti-parkinsonian, and anti-microbial activities of aqueous and ethanolic extracts of leaves of *V. leucoxylon* have studied the anti-inflammatory and wound healing properties of the crude alcoholic extract of the leaves in acute inflammation model. The roots and bark are astringent, and the roots are reported to be used as a febrifuge (table no. 2). Wood is mostly used for constructional and general purposes and for cartwheels, especially in Tamil Nādu. Owing to its attractive color, small and regular pores, and the broad and conspicuous medullary rays showing very small flecks of silvery grain, the wood is recommended for decorative cabinets or other work (The Wealth of India, Raw material)" (Makwana, H. G., *et al*., 1994, Althaf Faimum, D., *et al*., 2013, Venkateswarlu, G., Bodla, R., 2024). Chemical structures of isolated phytoconstituents from *Vitex leucoxylon* plant were presented in figure no.3.

Scientific classification:

Kingdom: Plantae

Class: Tracheophytes

Class: Angiosperms

Class: Eudicots

Class: Asterids

Order: Lamiales

Family: Lamiaceae

Genus: Vitex

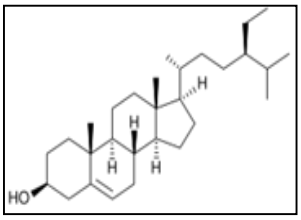
Species: *V. leucoxylon*

Binomial name: *Vitex leucoxylon* L.f.

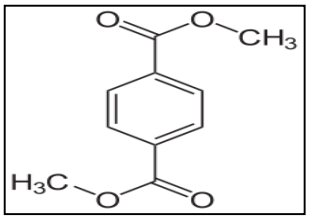
Fig.No.3: Photograph of *Vitex leucoxylon* plant

Table. No.2: List of phytochemicals isolated and biological activities of *Vitex leucoxylon*

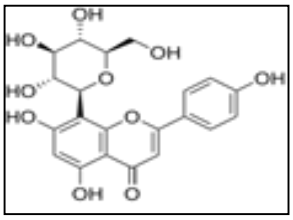
Species	Constituents isolated	Biological activity
Vitex leucoxylon	β-sitosterol, dimethyl terephthalate, vitexin, isovitexin, agnuside, aucubin	Hypoglycemic and anti-inflammatory properties, asthma, pox wounds, fever, insecticides, preserve food grains. inhibiting the increase of blood sugar. relieving headaches and catarrh. Antioxidant, anticancer, anti-psychotic, anti-depressant, analgesic, anti-inflammatory, anti-parkinsonian, and anti-microbial astringent, febrifuge.



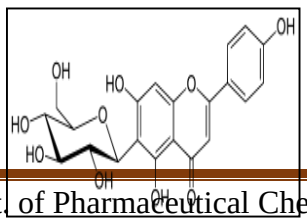
β-sitosterol



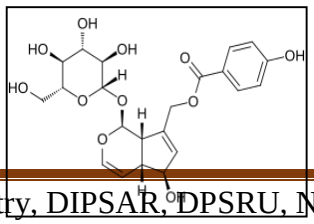
dimethyl terephthalate



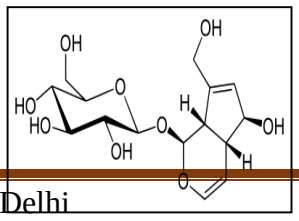
vitexin



isovitexin



agnuside



aucubin

Figure No. 4: Chemical structures of isolated phytoconstituents from *Vitex leucoxylon* plant

2. *Strychnos colubrina*:

Strychnos colubrina, (figure no.5) an endemic tree in peninsular India, is a large, climbing woody shrub with bifid tendrils. Its ovate-elliptic leaves are undulate or entire, acute or acuminate, rounded at the base, 3-nerved, coriaceous, and shiny. Flowers grow in cymes with corolla tube and lobes of equal length, and a woolly throat. The hirsute ovary and style measure 2.5-3 mm across. Creamy white flowers are 2-3 cm long. Axillary and old wood cymes produce globose orange-yellow berries, 1-1.8 cm across, with crustaceous pericarp. Seeds are 1-3, circular, flat, and 0.6-1.2 cm in diameter. Flowering and fruiting occur from September to November. The roots used in dyspepsia, intermittent fevers, malaria, swellings in chicken pox, joint discomfort, and diarrhoea, snake bite. The fresh leaves used in tumor and the bark is used as a febrifuge, dyspepsia, intermittent fever, and malarial cachexia. Fruits used in the treatment of mania. The stem decoction is used as a therapy for whooping cough, the dried leaf powder is smoked to alleviate asthma, the leaf paste is applied to cleanse scars from pox wounds, the root bark is employed to lower fever, fresh twigs serve as insecticides, and the dry leaf powder is utilised to preserve food grains.

Native healers also suggest using leaf extract to prevent the rise in blood sugar levels. It is employed as a traditional remedy for curing headaches and catarrh. The pharmacological studies exposed anti-depressant, analgesic, anti-inflammatory, anti-parkinsonian, and anti-microbial activities (table no.3). The anti-inflammatory and wound healing activities of aqueous and ethanolic extracts of *Strychnos colubrina* leaves have been examined using an acute inflammation paradigm (Sudhira, L., *et al*., 2015, Preethimol, F., & Suseem, S. R. 2022). Chemical structures of isolated phytoconstituents from *Strychnos colubrina* plant were presented in figure no:5.

Scientific classification:

Kingdom: Plantae

Class: Tracheophyta

Class: Magnoliopsida

Order: Gentianales

Family: Loganiaceae

Genus: Strychnos

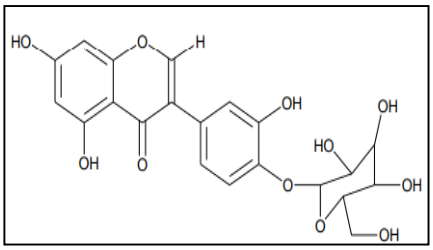
Species: *Strychnos colubrina*

Binomial name: *Strychnos colubrina* L.

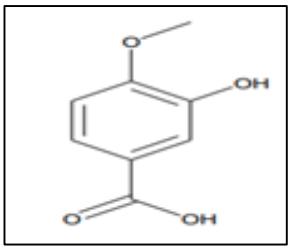
Figure No.5**:** Photograph of *Strychnos colubrina* Plant

Table. No. 3: List of phytochemicals isolated and biological activities of *Strychnos colubrina*

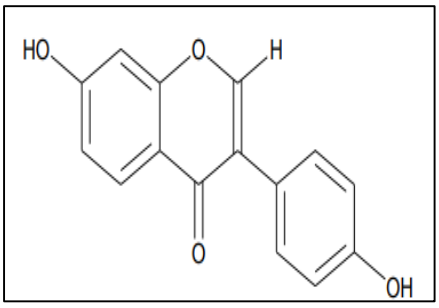
Species	**Constituents isolated**	**Biological activity**
Strychnos colubrina	(5, 7 – dihydroxy – 3 - (3 hydroxy – 4 - (3, 4, 5 trihydroxy – 6 - (hydroxymethyl) tetrahydro pyran – 2 – yl oxy) phenyl) chromen – 4 – one, Isovanillic acid (3 - Hydroxy, 4 methoxybenzoic acid), (7 – hydroxyl – 3 - (4 - hydroxyphenyl) – 4 H – 1 – benzopyran – 4 - one)	Dyspepsia, intermittent fevers, malaria, swellings in chicken pox, joint discomfort, and diarrhoea, snake bite. tumor febrifuge, dyspepsia, anticancer, antioxidant, intermittent fever, and malarial cachexia, mania. anti-inflammatory



5, 7 – dihydroxy – 3 - (3 hydroxy – 4 - (3, 4, 5 trihydroxy – 6 - (hydroxymethyl) tetrahydro pyran – 2 – yl oxy) phenyl) chromen – 4 - one



Isovanillic acid



(7 – hydroxyl – 3 - (4 - hydroxyphenyl) – 4 H – 1 – benzopyran – 4 - one)

Figure. No:6 Chemical structures of isolated phytoconstituents from *Strychnos colubrina* plant

3. *Boswellia ovalifoliolata* Balakr. and Henry (Burseraceae)

Boswellia ovalifoliolata Bal & Henry (Burseraceae) (figure no.7) is an endemic, endangered medicinal tree to Tirumala hills. It is commonly found growing on the foothill slopes of Tirumala Forest. The species is distributed extensively through to an altitude of about 300m from Vinayaka temple northwards to north-east and extending west and south-west to the temple up to galigopuram I. Leaflets 2-6 pairs, ovate-oblong, glaucous beneath, flowers greenish white, in much branched panicles. Deciduous trees with bark greyish, smooth, exfoliating in thin flakes, branchlets red colored, glabrous, leaves imparipinnate, leaflets 5-13, coriaceous, ovate or elliptic, glabrous, glaucous beneath, entire, obtuse, secondary nerves 8-10 pairs, reddish, sessile, flowers greenish white, in much branched panicles, calyx 5 toothed, petals 6, imbricate, disc annular, stamens 10, ovary 3- locular, ovules 2 per locule, pendulous, style short, stigma capitate drupes trigonous, pyrenes bony, seeds winged and compressed. Flowering and fruit season is March-June. Traditionally it is used as stem- stomach ulcers, diabetes, leaf- mouth & throat ulcers, gum- dysentery, inflammations, joint pains, ulcers, arthritis, amoebic dysentery, hydrocele, diabetes. Extracts of *Boswellia ovalifoliolata* exhibit

hypoglycemic and anti-inflammatory properties (table. no. 10). The stem decoction is used as a treatment for whooping cough, the dried leaf powder is smoked to alleviate asthma symptoms, the leaf paste is applied to cleanse scars caused by pox wounds, the root bark is used to lower fever, fresh twigs serve as pesticides, and the dry leaf powder is utilised to preserve food grains (Ratnam, K. V., & Bhakshu, L. M. 2023, Mahesh, B. U., *et al*., 2014). Chemical structures of isolated phytoconstituents from *Boswellia ovalifoliolata* plant were presented in figure no.7

Scientific classification:

Kingdom: Plantae

Class: Tracheophyta

Class: Magnoliopsida

Order: Sapindales

Family: Burseraceae

Genus: Boswellia

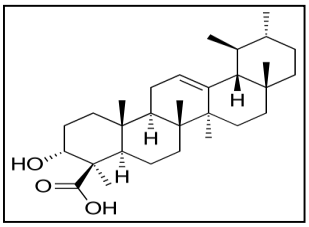
Species: *Boswellia ovalifoliolata*

Binomial name: *Boswellia ovalifoliolata* N.P. Balakr & A. N. Henry

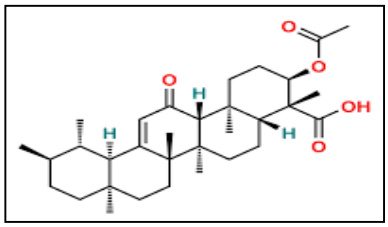
Figure.No.7 Photograph of *Boswellia ovalifoliolata* plant

Table. No. 4: List of phytochemicals isolated and biological activities of *Boswellia ovalifoliolata*

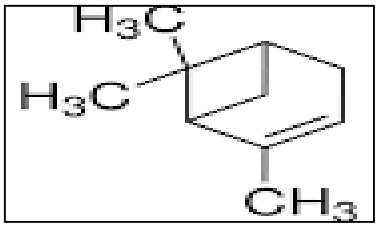
Species	Constituents isolated	Biological activity
Boswellia ovalifoliolata	beta-boswellic acid, acetyl-11-keto-beta-boswellic acid, acetyl-boswellic acid, alpha-pinene, limonene, beta-caryophyllene, kaempferol and quercetin	stomach ulcers, diabetes, antioxidant, anticancer, mouth & throat ulcers, dysentery, inflammations, hypoglycemic



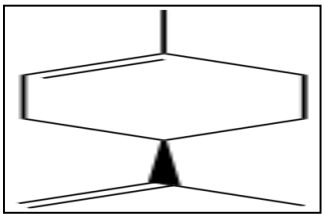
beta-boswellic acid



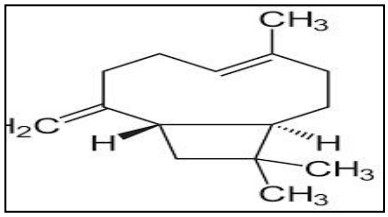
acetyl-11-keto-beta-boswellic acid



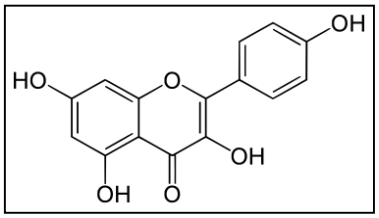
alpha-pinene



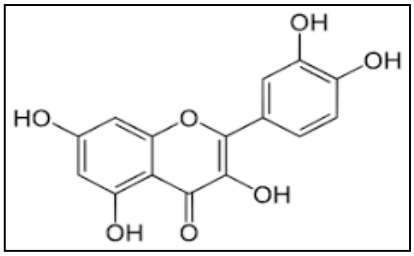
Limonene



beta-caryophyllene



kaempferol



quercetin

Figure. No:8 Chemical structures of isolated phytoconstituents from *Boswellia ovalifoliolata* plant

4. MATERIALS & METHODS

4.1. Materials and Methods:

All chemicals and solvents (hexane, toluene, chloroform, ethanol) were procured from Sigma-Aldrich Company and used.

4.1.1 Procurement of Plant Material:

For the present study, the leaves of *Vitex leucoxylon*, *Strychnos colubrina*, and *Boswellia ovalifoliolata*, have to be procured from Eastern ghats, Seshachalam hills, Tirupati, Andhra Pradesh. The plant materials were identified and authenticated by Dr. M. Sankararao, Scientist-D, Botanical Survey of India (BSI), Hyderabad, Telangana-500095.

4.1.2 Drying and Size Reduction:

After identification and authentication, leaves of *Vitex leucoxylon*, *Strychnos colubrina*, and *Boswellia ovalifoliolata*, were subjected to size reduction to a coarse powder by the grinder. The powder was stored in a tightly packed polythene bag until use.

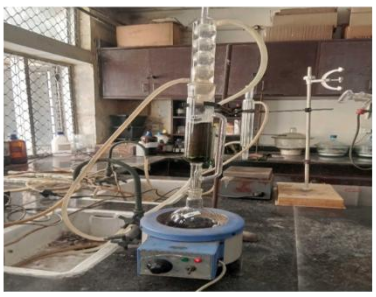
4.1.3 Extraction:

Powdered materials were charged into the Soxhlet apparatus for successive hot continuous extraction using the following solvents i.e., hexane, toluene, chloroform, ethanol and hydroalcoholic (1:1).

The plant material was extracted with each solvent until extraction was completed (about 40 cycles). The powdered material was air-dried before extraction with the successive solvent. A vacuum rota evaporator would concentrate both solvent extracts to obtain the crude extractive. The color, consistency, and percentage extractive values were calculated (figure no.9).

1. *Vitex leucoxylon*

2. *Strychnos colubrina*



3. *Boswellia ovalifoliolata*

VACUUM ROTA EVAPORATOR

Figure. No:9. Preparation of extracts by Soxhlet extraction and dried with rota evaporator

4.1.4 Phytochemical extraction:

The dried plant leaf powder was weighed accurately, and the required amount was placed in a Soxhlet extractor (Altemimi, A., *et al*., 2017) with a 1-liter capacity. A series of solvents with varying polarities, starting from non-polar (hexane) to polar (hydroalcohol, 1:1 mixture of water and alcohol), were used for successive extraction. The solvents used were hexane, toluene, chloroform, ethanol, and hydroalcohol.

After the extraction, the resulting crude extract was subjected to rotary evaporation to remove the solvents. The residue was then dried at room temperature. The dried extract was weighed, and the percentage yield of the extract was calculated using a specific formula. The formula for calculating the percentage extraction is also.

% Extraction = ((M1 – M2) / M1) X 100

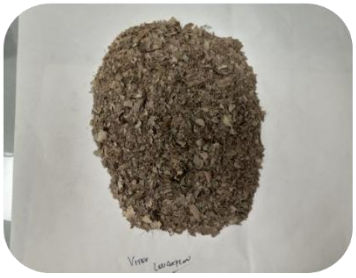
Where, M1 = Mass in grams of the sample before extraction.

M2= Mass in grams of sample after extraction.

Table. No:5. Overall data of plant materials and solvents used for extraction:

S. No	**Plant materials**	**Weight (gm)**	**Time (hrs)**	**Solvent (ml)**	**Boiling Points (°C)**				
					H	**T**	**$CHCl_3$**	**EtOH**	**HA**
1	*Vitex leucoxylon*	74.41	48	350	68.7	110.6	61.2	78.3	100:78.3
2	*Strychnos colubrina*	59.00	48	350	68.7	110.6	61.2	78.3	100:78.3
3	*Boswellia ovalifoliolata*	121.10	48	350	68.7	110.6	61.2	78.3	100:78.3

1. *Vitex leucoxylon*



2. *Strychnos colubrina*

3. *Boswellia ovalifoliolata*

Figure No:5. coarse powder materials of the selected medicinal plants

4.1.5 Preparation of Extracts:

Powdered all plant materials are weighed and placed in soxhlet apparatus.

For soxhlet extraction using solvents (hexane, toluene, chloroform, ethanol, and hydroalcohol)

The solvent was recovered using a rotary vacuum evaporator.

Obtained the residues.

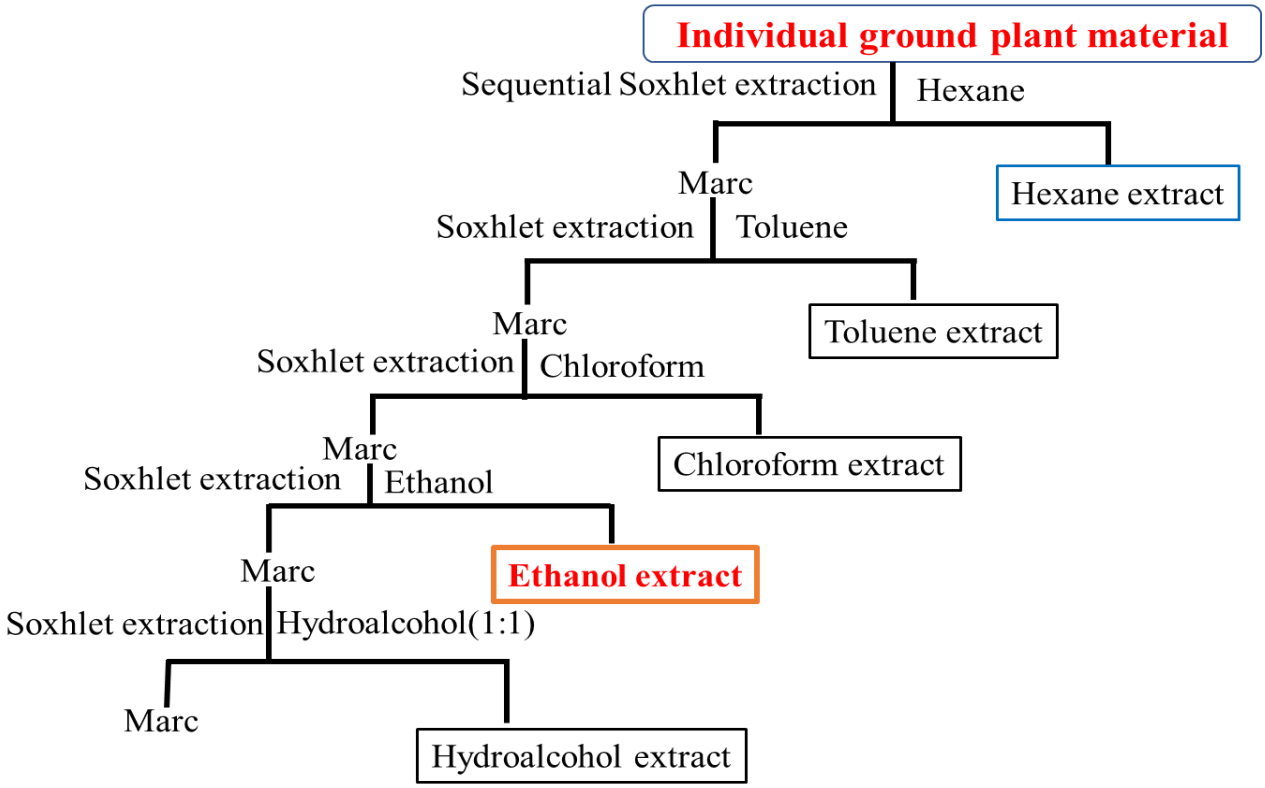


Figure No:10. Flow chart of extraction of the selected plant materials

4.1.6. PHYTOCHEMICAL SCREENING FOR PHYTOCONSTITUENTS:

4.1.7 Qualitative phytochemical screening of plant-extracted materials for the detection of phytochemicals.

The plant may be considered a biosynthetic laboratory for many compounds like alkaloids, flavonoids, phenols, steroids, triterpenoids, etc., that exert a physiological effect. The compounds that are responsible for the therapeutic effect are usually secondary metabolites. A

systemic study of a crude drug embraces consideration of primary and secondary metabolites derived from plant constituents (Rajasekar, A. 2015).

The extracts were subjected to preliminary phytochemical screening to detect various plant constituents like alkaloids, diterpenoids, sterols, phenolics, glycosides, etc. The phytochemical analysis of extracts was carried out using the following tests.

Solvent-free extracts of hexane, toluene, chloroform, ethanol, and hydroalcoholic were stirred with little dilute HCl and filtered. The filtrate was subjected to the following tests (Table No.6).

Table No:6. Qualitative phytochemical screening of plant-extracted materials for the detection of phytochemicals

Phytochemical	**Name of test**	**Methodology**	**Result(s)**
Alkaloids	Mayer's test	Few ml of filtrate + 2 drops of Mayer's reagent (Potassium mercuric iodide solution) along the sides of the test tube.	Formation of white or cream color precipitate.
	Wagner's test	Filtrate + a few drops of Wagner's reagent (Iodine-potassium iodide solution)	Formation of a reddish-brown precipitate.
	Hager's test	Few ml of filtrate + 1 or 2 ml of Hager's reagent (Saturated solution of picric acid)	Formation of yellow precipitate.
	Dragendorff's test	Few ml of filtrate + 1 or 2 ml of Dragendorff's reagent (Potassium bismuth iodide solution)	Formation of the reddish-brown or orange-brown precipitate.
	Tannic acid test	few ml of filtrate + a few drops of tannic acid solution.	Formation of a buff-colored precipitate.
	Lead acetate test	To the alcoholic solution, add a few drops of 10% lead acetate solution.	Formation of yellow precipitate.

Flavonoids	Shinoda test	To the test solution, adds a few magnesium turnings and conc. HCl, drop-wise.	Appears pink, scarlet, crimson red, or occasionally green to blue color.
	Alkaline reagent test	2 ml of 2.0% NaOH mixture was mixed with aqueous plant crude extract.	Firstly, the concentrated yellow color was produced, which became colorless when added two drops of diluted acid to the mixture.
	Zinc Hcl test	A mixture of zinc dust and concentrated hydrochloric acid was added to test the flavonoid sample solution.	Results in red color.
Tannins	Ferric chloride test	To the alcoholic solution, add 5% $FeCl_3$ solution.	An intense green, purple, blue/black color developed.
	Lead acetate test	To the sample, add 10% lead acetate solution.	Precipitate formed.
Phenols	Ferric chloride test	To the alcoholic solution, add 5% $FeCl_3$ solution.	Formation of green color.
	Lead acetate test	Add 10% of the lead acetate solution to the sample.	A white precipitate was formed.
Steroids and triterpenoids	Libermann-Buchard test	Treat the extract with a few drops of acetic anhydride, boil, and cool. Then add conc. H_2SO_4 from the sides of the test tube.	The Brown ring is formed at the junction of two layers. The upper layer turns green, which shows the presence of steroids.

			The formation of deep red color indicates the presence of triterpenoids.
	Salkowski test	Treat the extract with a few drops of conc. H_2SO_4 (or) chloroform solution of sample + Conc. H_2SO_4.	The red color at the lower layer indicates the presence of steroids. The formation of a yellow-colored lower layer indicates the presence of triterpenoids.
Anthocyanins	Anthocyanins test:	Sample + 2ml of 2N HCl.	Firstly, its appearance of pink-red color after adding ammonia turns to purplish-blue color.
Glycosides	Glycoside test	0.5 mg of bark extract was dissolved in 1 ml of H_2O & then an aqueous NaOH solution was added.	Formation of yellow color.
Saponins	Honeycomb test	0.5mg of extract was taken in a test tube & few drops of 5% sodium bicarbonate solution were added. The mixture was shaken vigorously& kept for 3 mins.	The formation of honeycomb-like froth shows the presence of saponins.
	Foam test	0.5mg of extract was diluted with 20ml distilled H_2O &	The foam formation to a length of 1cm

		shaken well in a graduated cylinder for 15mins.	indicated the presence of saponins.
Anthraquinone	Bontrager's test	0.5gm of the extract was taken into a dry test tube & 5 ml of chloroform was added and shaken for 5min. The extract was filtered & the filtrate was shaken with an equal volume of 10% ammonia solution.	A pink-violet/red color in the lower layer indicates the presence of anthraquinone.

4.1.8 Extraction and isolation of Inugalactolipid-A from *Vitex leucoxylon*.

The plant material (1.550 kg) was extracted with ethanol (4 L) and concentrated over rotary evaporator to get crude (138 g, extractive value 8.9%) material. The crude erxtract (138 g) was fractioned into 13 fractions (each fraction collected to 500ml) with polarity based solvent system i.e., DCM, DCM-MeOH, MeOH over a silica gel mesh size (100-200 mesh) loaded in a open column. Later fraction 10 (5.286 g) was sub fraction into 56 fractions over silica gel mesh size 10-200 with the solevnt system of chloroform and methanol. Here sub fractions of fraction 10 and 11 (1.72g) was loaded into column containg Sephadex-20 with methanol as a solvent, it resulted into 3 fractions. Entire process was monitored by TLC after evaporation of collected fractions and spotted on TLC with solvent system of 2% DCM/MeOH, visualized after spraying with anisaldehyde reagent. After esterification of fraction 1, Inugalactolipid-A was isolated (Rasul, M. G. 2018).

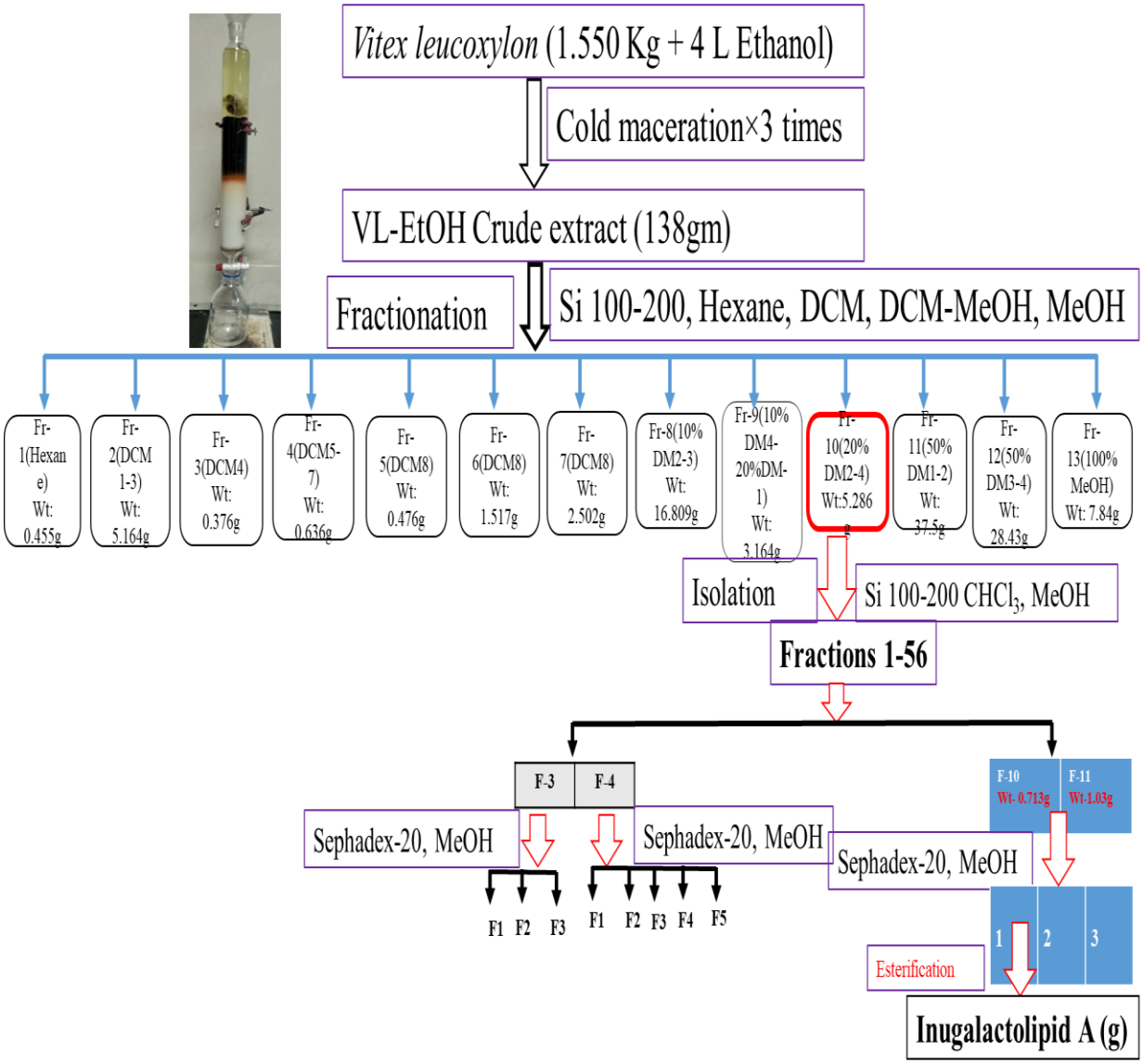


Figure No:11. Extraction and isolation of Inugalactolipid-A from *Vitex leucoxylon*

4.1.9 Extraction and isolation of Cirsimarin from *Stryhnos colubrina*.

The plant material (0.980 kg) was extracted with ethanol (2 L) and concentrated over rotary evaporator to get crude (85 g, extractive value 8.6%) material. The crude erxtract (85 g) was fractioned into 9 fractions with polarity based solvent system i.e., DCM, DCM-MeOH, MeOH over a silica gel mesh size (100-200 mesh) loaded in a open column. Later fraction 5 (12.219 g) centrifuged and isolated the lupeol (0. 233g) then fraction-5 (11.023g) was loaded over silica gel mesh size 100-200 and run with the solevnt system of 5% ethylacetate:hexane, isolated compounds were process was monitored by TLC after evaporation of collected fractions and spotted on TLC with solvent system of 2% EtOAc/hexane, visualized after spraying with anisaldehyde reagent of beta sitosterol (0.050g), urosolic acid (0.07g) and Oleonolic acid

(0.1g). fraction 7 and 8 (6.720g) was centrifuged with rpm 4000 and entire process was monitored by TLC after evaporation of collected fractions and spotted on TLC with solvent system of 1% hexane/EtOAc, visualized after spraying with anisaldehyde reagent, cirsimarin (1.456g) was isolated (Visht, S., & Chaturvedi, S. 2012).

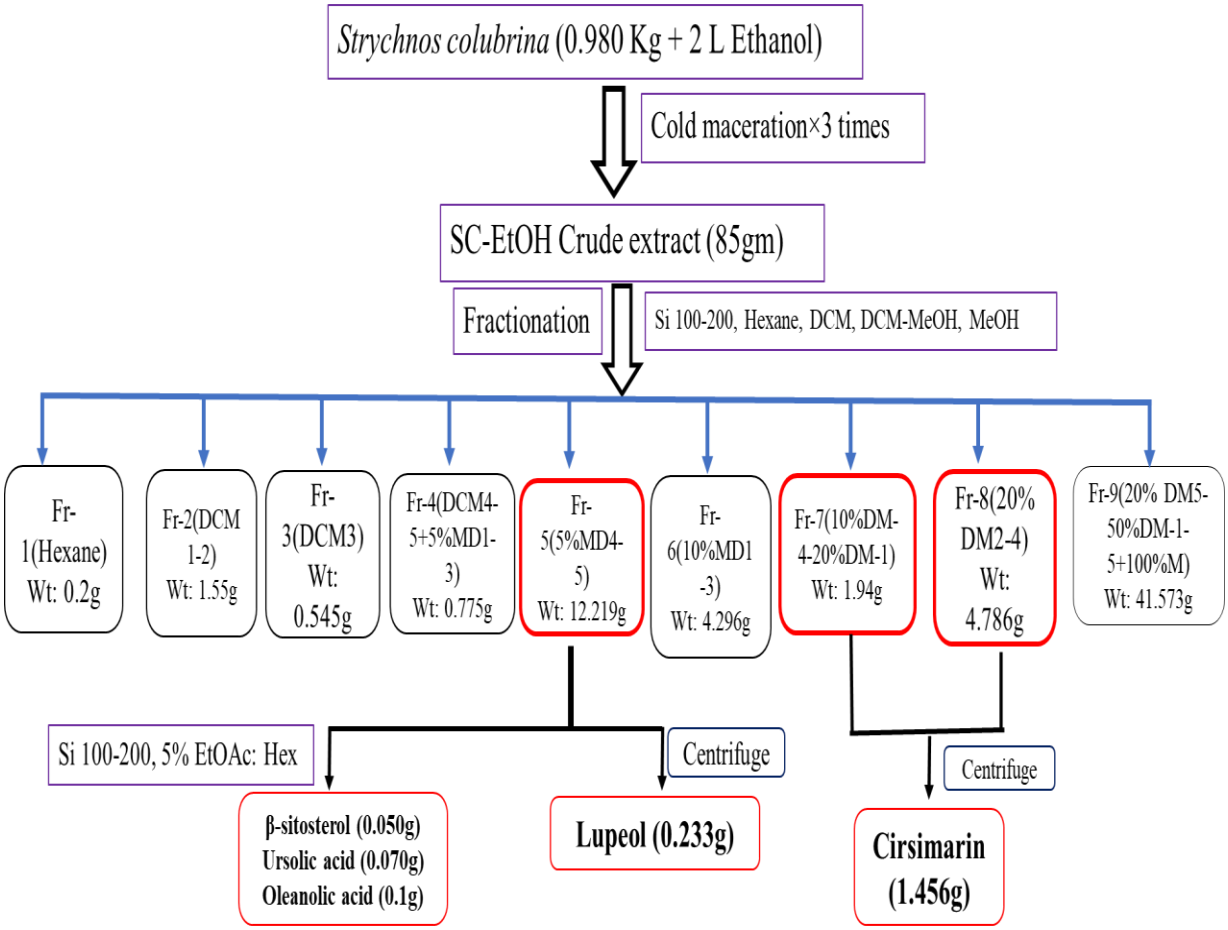


Figure No:12 Extraction and isolation of Cirsimarin from *Stryhnos colubrina*.

4.2 Extraction and isolation of Myrecitin-7-Glucoside from *Boswellia ovalifoliolata*.

The plant material (2.750 kg) was extracted with ethanol (6 L) and concentrated over rotary evaporator to get crude (470 g, extractive value 17.09%) material. The crude erxtract (470 g) was separated into solid (230 g) and liquid portions. 230 g of sample was loaded into column, HP-20 used as a stationary phase with the solvent system of water, methanol:water, methanol with increased the polarity and collected 21 fractions. In further process step 20.7 g of fraction-12 was soluble in water for this ethyl actetate, butanol and water and separetd into ethyl actetate, butanol and water fraction by separating funnel. Finally butanol fraction (8.97 g) run through Sephadex 20 column, methanol used as solvent, the process was monitored by TLC after evaporation of collected fractions and spotted on TLC with solvent system of 4%

MeOH/$CHCl_3$, visualized after spraying with anisaldehyde reagent here we isolated the Mycercitin-7-Glucoside (Colegate, S. M., & Molyneux, R. J. (Eds.). 2007).

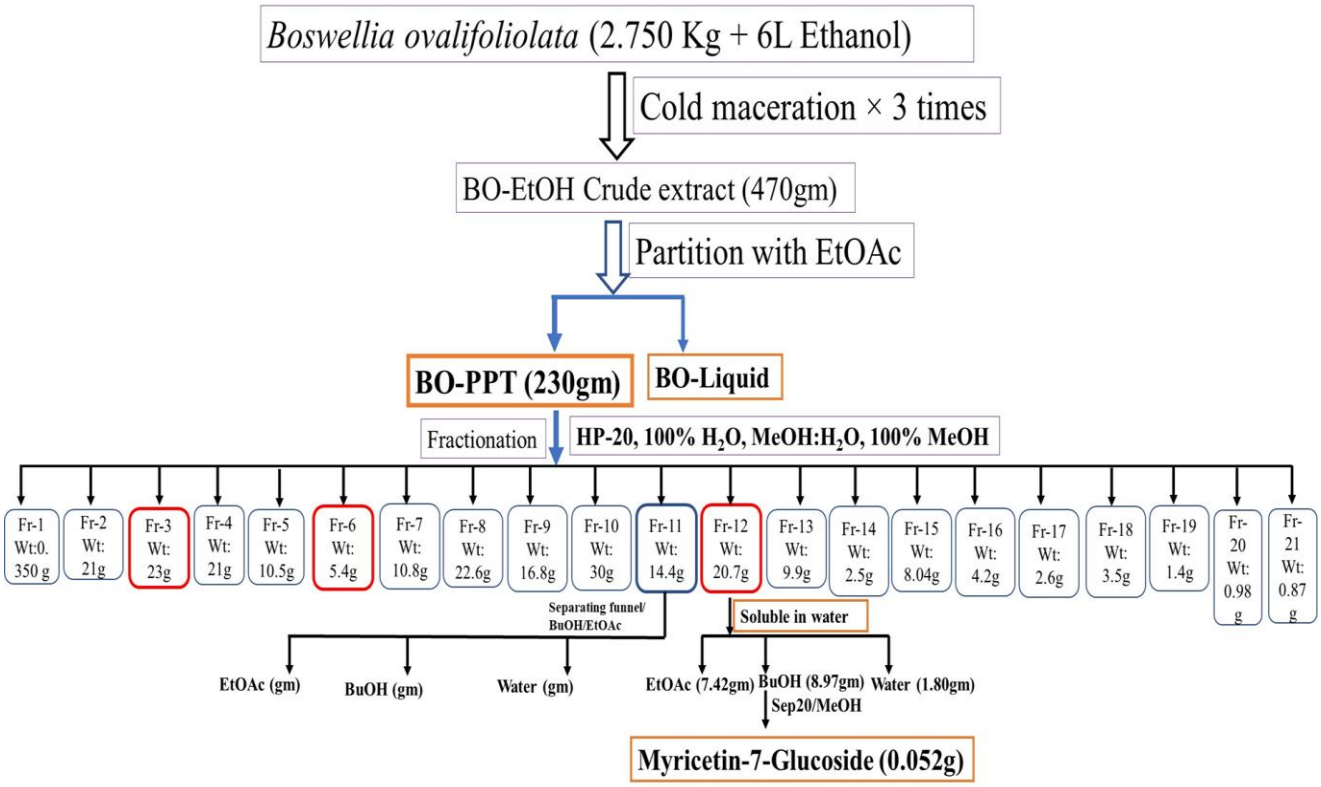


Figure No:13. Extraction and isolation of Myrecitin-7-Glucoside from *Boswellia ovalifoliolata*.

4.2.1 Analysis of extracts, fractions and isolated compounds

4.2.2 TLC (Thin Layer Chromatography)

Thin Layer Chromatographhty was carried out on precoated silica gel plates 60 F_{254} plates (Merck, India) with 1 mm film thickness. Dragendorff and anisaldehyde– H_2SO_4 reagents were used to visualize spots on TLC plates.

4.2.3 High performance thin layer chromatography (HPTLC):

HPTLC fingerprinting of various extracts was performed using CAMAG HPTLC analysis. The stationary phase consisted of activated and pre-washed TLC aluminum plates, measuring 20 x 10 cm, and pre-coated with silica gel 60F-254 (E. Merck). For the mobile phase, a mixture of 1% ethyl acetate in hexane, 2% ethyl acetate in toulene was used during the analysis. A deuterium and tungsten lamp served as the radiation source for the analysis. The CAMAG twin trough chamber was prepared by pre-saturating it with the mobile phase for 20 minutes. The slit dimensions were set to 5.00 x 0.45 mm, with a data resolution of 100 µm/step. Detection was conducted at a wavelength of 254 nm using a densitometric scanner (CAMAG TLC scanner) operated with Win CATS software, at a scanning speed of 20 mm/s. Samples were

automatically injected into the system, and 10 µl of each sample was applied to the plate in the form of 7 mm-wide bands using an automated sample applicator or a µl syringe and calculated the R_f value by using the following formula (Hamilton, Switzerland) (Attimarad, M., *et al*., 2011)." The Rf value of a compound is equal to the distance traveled by the compound divided by the distance travelled by the solvent front (both measured from the origin).

4.2.4 FTIR Analysis

FT-IR spectroscopy was performed using Spectrum Two, PerkinElmer FT-IR spectrometer using KBr disc method for spectral measurement. The sample pellet of the extracts and isolated compounds was prepared by mixing the required amount of dried extract with the KBr salt using a mortar and pestle and pressurizing it with KBr press to make the pellet. The sample pellet was scanned, and the IR spectrum was recorded in the range of 550 to 4000 cm^{-1} (Oliveira, R. N *et al*., 2016).

4.2.5 HPLC Analysis

HPLC analysis was performed for isolated compounds (100µg/mL) on a HPLC Nexera Shimadzu connected with SPD-M20A diode array detector. The analysis used a Cyano RP-18, column with dimensions 100 × 4.6mm,3um particle size. The mobile phase A-Buffer (0.1% Formic acid in Water) B- Organic (Methanol) respectively. A gradient elution started with 90%A and 10%B, holding till 5 mins. Then, the mobile phase gradient was changed to 65%A and 35%B from 5 to 20 minutes, keeping this ratio till 40 mins. Then, changed to the initial ratio from 20 to 30 minutes and hold it for ten more minutes. So, the total run time of the analysis is 40 mins, maintaining a constant flow rate of 0.6ml/min. The PDA ranges between 190-800nm, and the final chromatogram was extracted at a λmax of 254nm/366nm. A constant column temperature was maintained at 40^{o}C (Engida, A. M *et al*., 2013).

4.2.6 GC/MS Experimental System and Measurements

The GC/MS analysis was performed for all the extracts (10µg/mL) using an instrument Shimadzu TQ8040 NX GS-MS which was coupled with silica capillary column TG-5-MS (dimensions-30.0 m×0.25 mm, film thickness 0.25µm). For GC/MS detection an electron impact ionization system with ionizing energy of 70 eV with a scanning mass range was set at 29–400 (*m/z*) and helium carrier gas with a flow rate of linear velocity (41.4 cm/s) was used. Before the commencement of the phytochemical analysis procedure, the oven temperature was initiated at 60°C for 1 min. After the end of this preparatory phase is programmed to increase up to 300°C at the rate of 3°C/min, and later maintained isothermally for 15 min. The injector port, ion source, and detector temperature were set at 280°C, 220°C, and 280°C, respectively. The total run time for GC was 20 min. The National Institute of Standards & Technology

(NIST) Library database was used to retrieve the components' names, molecular weights, and structures.

4.2.7 Mass spectroscopy (MS):

All the isolated compounds (50µg/mL) ESI-MS spectra were recorded on Agilent 1100LC-QTOF and HRMS-6540-UHD machines. A 6410 B triple quad LC/MS system from Agilent was used for the detection, which is a hybrid triple quadrupole mass spectrometer equipped with Turbo V sources. The analyses were performed using an electrospray ionisation (ESI) source in positive and negative modes. The operation conditions were as follows: scan rangeof 100–600 amu, V charging 2000 V, ion source temperature 325 °C, nebulizer 60 psi, gas flow 12 L/min, capillary voltage 4000, collision gas nitrogen, dwell time of 50 ms and a step size of 0.1 amu. Nitrogen was used in all cases. The results of the precursor ion, product ionand collision energy (CE, eV) are shown in section S14 of supporting information. The dwell time was setat 60 ms in positive mode. Agilent Mass Hunter software (version B.04.00) was used for data acquisition and processing. Dictionary of Natural Product (CRC, Chapman and Hall, London, 2011 on DVD) and online version was used as dereplication database for isolated compounds (Pan, X., *et al*., 2010).

4.2.8 High resolution mass spectroscopy (HRMS) analysis:

Isolated compound of cirsimarin (50µg/mL) was recorded by High resolution mass spectra were obtained on Agilent 6540 (Q-TOF) mass spectrometer, in the electrospray (ESMS) mode. Optical rotation was measured on a Perkin Elmer 341 polarimeter in a 1 dm cell at 25°C (Alvarez-Rivera *et al*., 2019).

4.2.9 Nuclear magnetic resonance spectroscopy (NMR) analysis:

All the isolated compounds (20µg/mL) of ^{1}H NMR spectra were recorded (BruckerAvance) at 400 and 500 MHz and ^{13}C NMR at 100 and 125 MHz in CDCl3, CD3OD and Pyr-d5. The chemical shifts values are reported in δ (ppm) units and coupling constants values in hertz. Tetramethylsilane (TMS) was used as an internal standard. The following abbreviations were used to explain multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad. Dry Dimethyl formamide was purchased from Sigma Aldrich while other regents were purchased commercially and used without further purification (Chauthe, S. K., *et al*., 2012).

4.3 Evaluation of Anticancer activity

4.3.1 Determination of *invitro* cytotoxicity using human cancer cell lines

4.3.2 Chemicals

Roswell Park Memorial Institute medium (RPMI-1640), dimethyl sulfoxide (DMSO), fetal bovine serum (FBS), phosphate buffer saline (PBS), minimum essential medium (MEM), glacial acetic acid, sulphorhodamine Blue (SRB), mitomycin C, camptothecin, paclitaxel,

trypsin, and gentamycin were obtained from Sigma Aldrich, USA. Glacial acetic acid, isopropyl alcohol, and trichloroacetic acid were procured from Sisco Research Laboratories, Mumbai. Ethylenediamine tetraacetic acid, disodium salt (EDTA), and tris buffer were from HiMedia Laboratories, Mumbai.

4.3.3 Apparatus

96-well flat bottom tissue culture plates (Iwaki, Cat No, 3860-096), cryovials (Nunc, Cat No. V 7384); 15 or 50 ml radiation sterilized centrifuge tubes (Tarsons Products Pvt. LTD, Cat NO. 546020/546040); microcentrifuge tubes (Tarsons Products Pvt. LTD); micropipettes (Tarsons Products Pvt. LTD); sterile disposable syringes (10ml. 5mI, 2ml) (Dispovan, Hindustan Syringes, and Medical Devices LTD); syringe driven filter unit (0.22μ) (Millipore, Cat no. SLGP033RS, Carrigtwohill, Ireland); micropipette-tips (Tarsons Products Pvt. LTD); pipette dispenser (Accu-jet g pro. Brand, Germany); Tissue culture flasks (T-25 — 75 & T-150 cm^2) (Nunc. Cat no, 156499, Denmark) and cryo I°C freezing container or cryobox (Mr. Frosty, Sigma Chem. Co, USA) were used for the study.

4.3.4 Instruments

Carbon dioxide incubator (Heraeus, Germany), centrifuge (Beckman, USA). deep freezer (Model 702, Thermofisher), filtration assembly, weighing balance (Sartorius. Germany) hemocytometer (Sigma Chem. Co., USA), mechanical shaker (IKA Germany), microscope (Nikon, Japan), sunrise ELISA reader (Tecan, Switzerland). vertical laminar flow - clean air workstation (Klenzaids), liquid nitrogen container (Thermo Electron Corporation, USA) were used in the current study.

5.1 Cell lines used

In vitro anticancer screening of different extracts, fractions and isolated compounds from three medicinal plants was carried out against five human cancer cell lines as presented in Table No.7. Human cancer cell lines representing different tissues were selected and the mass doubling time of cell lines was such that enough cells were obtained for conducting assay.

Table No.7: List of the cell lines used for anticancer screening

S. No.	Tissue	Cell line	Doubling time (hr)	Cell density /100 µl	Medium	Positive control
1.	Breast	MCF-7	25.4	8000	RPMI	Doxorubicin
2.	Colon	HCT-116	17.4	7000	RPMI	5-Fluorouracil

3.	Lung	A-549	22.9	7500	RPMI	Paclitaxel
4.	Pancreas	MIA PaCa-2	40	10000	DMEM	Paclitaxel
5.	Glioblastoma	U251	23	10000	DMEM	Paclitaxel

liquid nitrogen for indefinite storage.

6.0 Preparation of test compound for *in-vitro* studies

6.1 Stock Solution

Dimethyl sulfoxide (DMSO) was used for the preparation of stock solution of the test sample. Stock solution of each test sample was prepared having 100 µg/mL (extracts, fractions and isolated molecules) concentration. In case of compounds, 20 mM of stock solutions were prepared atleast one day in advance. and kept at 4 ºC for future use.

6.2 Working Solution

An aliquot of the frozen stock solution was thawed at room temperature on the day of the assay. By diluting the stock solution with complete media, a working test solution of the extract or fraction (100 µg/mL) was prepared. Working solutions of 2.5, 5, 10, 20, 40 and 1 µM were prepared for the compounds.

6.3 Positive controls (standard drugs for cancer)

Positive controls were initially prepared with DW (Doxorubicin, 5–flurouracil, mitomycin-C) and DMSO (paclitaxel). They were further prepared in complete medium to obtain working test solutions.

6.4 Cellular Anti-proliferative activity

In 96 well flat bottom plates, the optimal cell density per well was seeded in order to perform the SRB assay, which was used to assess the cytotoxic potential of the potent drug. Human cancer cell lines namely colon cancer (HCT-116), Breast (MCF-7), lung cancer (A-549), Pancreatic (MIA PaCa-2), Glioblastoma (U251) were seeded 100µl/well in 96 well plates. The cells were exposed to test substances at varying concentrations (2.5, 5, 10, 20, and 40 µg/mL), which contained complete growth media as well as camptothecin as a positive control. The plates were incubated for 48 hours. Furthermore, ice-cold TCA was used to fix cells. Additionally, cells were fixed for one hour at 4ºC with ice-cold TCA. The plates were given three water rinses after one hour and then left to air dry. In the following step, 100µL of 0.4% SRB dye was applied, and plates were left at room temperature. After that, plates were washed once with 1% acetic acid and three times with water to remove the unbound SRB. The bound dye was solubilized after drying at room temperature by adding 100 µL of 10 mM Tris buffer (pH-10.4) to each well. To

dissolve the protein-bound dye, the plates were shaken for 5 minutes. Using Graph PAD Prism Software Version 5.0, OD was determined at 540 nm in a microplate reader (Thermo Scientific) (Houghton, P., *et al*., 2007).

6.5 Calculations

The cell viability was determined (Youssef, A. M., *et al*., 2022) by dividing average absorbance value of treated sample by the average absorbance value of control and multiplied by 100. Percent growth inhibition in presence of test material was calculated as under:

$$\text{Cell viability} = \frac{\text{Average of treated sample}}{\text{Average of control}} \times 100$$

% Growth inhibition = 100 - Cell viability.

6.6 Criteria for activity

The growth inhibition of 70% or above was considered active while screening extracts/fractions, but in testing of active ingredient/compounds at different molar concentrations, the growth inhibition of 50% or above was the criteria of activity.

6.7 Morphological studies using 4, 6-diamidino-2-phenylindole (DAPI) staining and Bright field microscopy.

Nuclear damage is a significant factor in determining cell apoptosis. DAPI dye is used to stain the nucleus to assess apoptosis determined by nuclear damage. HCT 116 cells were seeded @ 1×10^5 cells per well of a 6-well plate and were kept in an incubator overnight for the cells to attain their morphology. After 24 hours of incubation, cells were treated with Inugalactolipid, Cirsimarin and Myricetin-7-Glucoside at the concentrations of 2.5, 5, 10, and 20µg/mL along with camptothecin as a positive control at the concentration of 0.25µM and the plates were kept in the incubator for 48 hours. The cells were given PBS wash twice and were fixed with 4% paraformaldehyde (Sreelatha, S *et al*., 2011). DAPI dye was used for staining the fixed cells, and the stained cells were observed under the fluorescent microscope.

6.8 Assessment of mitochondrial membrane depolarization

The cytoplasmic release of apoptogenic stimuli causes loss of mitochondrial membrane potential which results in cell death. HCT 116 cells were seeded @ 1×10^5 into each well of a six-well plate, and the cells were left in the incubator overnight to develop their morphology. Cells were treated with Inugalactolipid, Cirsimarin and Myricetin-7-Glucoside at the concentrations of 2.5, 5, 10, and 20µg/mL along with camptothecin as a positive control at a concentration of 0.25µM, and the plates were left to incubate for 48 hours. PBS wash was given twice, and 1ml of 200 nM of Rhodamine-123 dye was added to each well and the plate was incubated for 15 minutes so as

to allow the cells to take up the dye. After 15 minutes, wells were washed with PBS thrice and 1ml of incomplete media was added to each well and images were taken under a fluorescent microscope (Agarwal, C., *et al*., 2002).

6.9 Determination of intracellular peroxides (ROS Assay)

An increase in the generation of reactive oxygen species is the primary indication of the apoptosis in a cell. HCT 116 cells were seeded at a density of 1×10^5 cells per well of a 6 well plate and were kept in the incubator overnight to attain their morphology. After 24 hours of incubation, cells were treated with the Inugalactolipid, Cirsimarin and Myricetin-7-Glucoside at the concentrations of 2.5, 5, 10, and 20µg/mL. After 48 hours of incubation, cells were washed with PBS and 10 µmol / L of DCFDA dye was added to each well for 20 minutes. Again PBS wash was given and observed under fluorescence microscope. H_2O_2 was used as positive control (Ezhilarasan, D., *et al*., 2019).

7.0 Statistical analysis

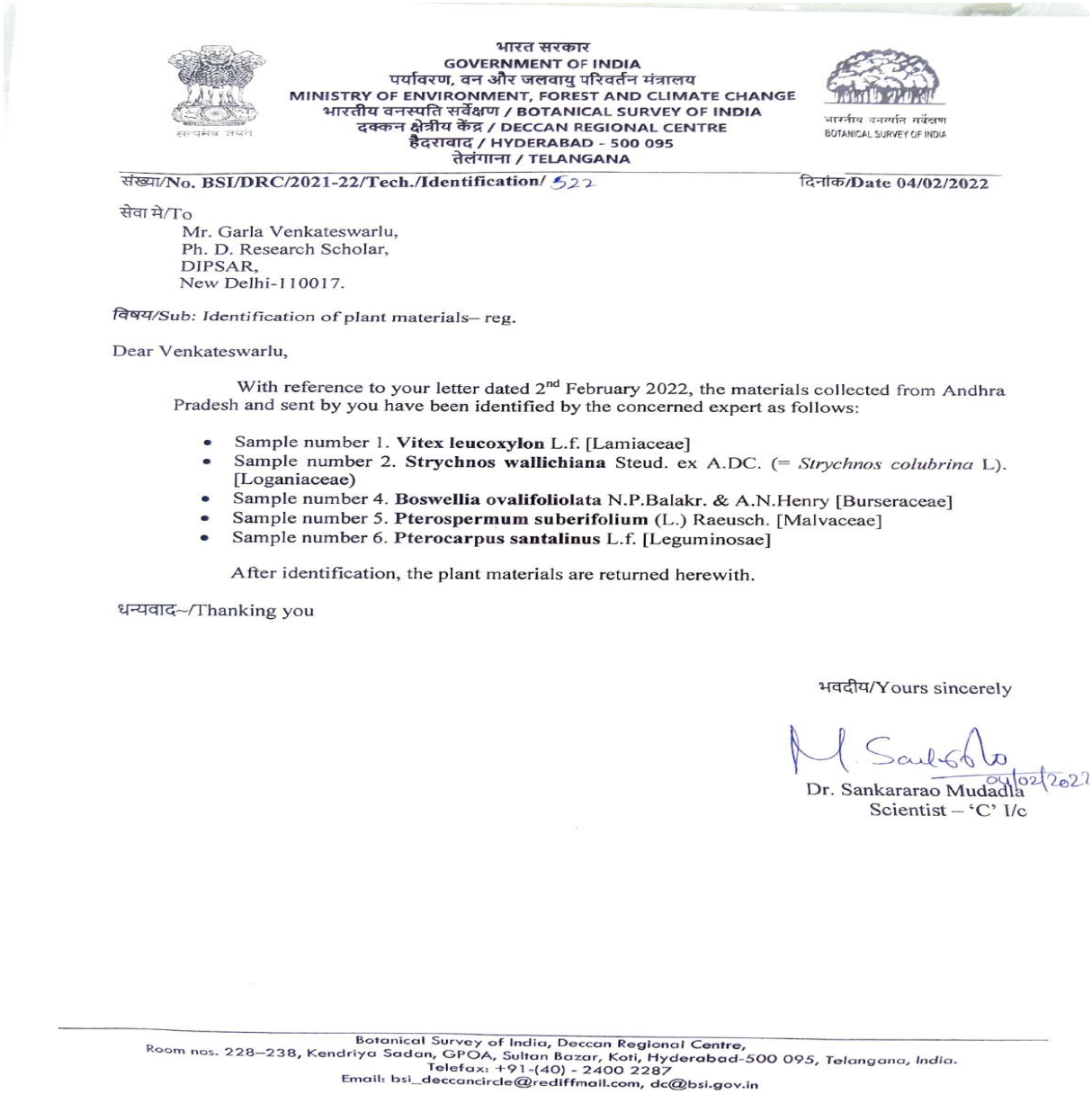
The experiments were conducted in triplicate and each dataset represents the mean of at least three independent experiments. Data were presented as Mean ± S.D. Data was analyzed by means of one-way analysis of variance (ANOVA) and post hoc differences between samples were determined by Tukey's test at $p \leq 0.05$ probability level, using R Studio software (version, 2021). IC_{50} value was determined using GraphPad Prism software version 5.0.

5. RESULTS AND DISCUSSION

5.1 Authentication of Plant Materials:

The plant leaves are gone and collected during the field visit to Seshachalam hills, Tirupati, Andhra Pradesh. The materials were identified and authenticated at "Botanical Survey of India, Hyderabad". The authentication certificates are enclosed.

Figure No. 13: Authentication certificate of medicinal plants.



भारत सरकार
GOVERNMENT OF INDIA
पर्यावरण, वन और जलवायु परिवर्तन मंत्रालय
MINISTRY OF ENVIRONMENT, FOREST AND CLIMATE CHANGE
भारतीय वनस्पति सर्वेक्षण / BOTANICAL SURVEY OF INDIA
दक्कन क्षेत्रीय केंद्र / DECCAN REGIONAL CENTRE
हैदराबाद / HYDERABAD - 500 095
तेलंगाना / TELANGANA

संख्या/No. BSI/DRC/2021-22/Tech./Identification/ 522 दिनांक/Date 04/02/2022

सेवा मे/To
Mr. Garla Venkateswarlu,
Ph. D. Research Scholar,
DIPSAR,
New Delhi-110017.

विषय/Sub: Identification of plant materials– reg.

Dear Venkateswarlu,

With reference to your letter dated 2nd February 2022, the materials collected from Andhra Pradesh and sent by you have been identified by the concerned expert as follows:

- Sample number 1. **Vitex leucoxylon** L.f. [Lamiaceae]
- Sample number 2. **Strychnos wallichiana** Steud. ex A.DC. (= *Strychnos colubrina* L). [Loganiaceae)
- Sample number 4. **Boswellia ovalifoliolata** N.P.Balakr. & A.N.Henry [Burseraceae]
- Sample number 5. **Pterospermum suberifolium** (L.) Raeusch. [Malvaceae]
- Sample number 6. **Pterocarpus santalinus** L.f. [Leguminosae]

After identification, the plant materials are returned herewith.

धन्यवाद–/Thanking you

भवदीय/Yours sincerely

04/02/2022
Dr. Sankararao Mudadla
Scientist – 'C' I/c

Botanical Survey of India, Deccan Regional Centre,
Room nos. 228–238, Kendriya Sadan, GPOA, Sultan Bazar, Koti, Hyderabad-500 095, Telangana, India.
Telefax: +91-(40) - 2400 2287
Email: bsi_deccancircle@rediffmail.com, dc@bsi.gov.in

5.2 Results:

5.3 Successive Hot Continuous Extraction:

The percentage yield and characteristics of the residues obtained by the Soxhlet hot continuous extraction of the dried coarse powdered different plant leaves of *Vitex leucoxylon, Strychnos colubrina,* and *Boswellia ovalifoliolata* with hexane, toluene, chloroform, ethanol, and hydroalcohol (1:1) are given in the table no.8.

Table No.8. The percentage yield and physical characteristics of *Vitex leucoxylon* plant leaf extracts.

S. No	Plant	Extract	Appearance	Consistency	Yield (%)
1	***Vitex leucoxylon***	Hexane	Light green	sticky, semisolid	0.82
		Toluene	Green	amorphous powder, solid	1.97
		Chloroform	Green,	amorphous powder, solid	1.048
		Ethanol	Green,	sticky, semisolid	5.03
		Ethanol: Water (1:1)	Light black,	non-sticky, powder, shiny particles	7.61
1	***Vitex leucoxylon***	Ethanol	Cold maceration		8.90

Table No.9. The percentage yield and physical characteristics of *Strychnos colubrina* plant leaf extracts.

S. No	Plant	Extract	Appearance	Consistency	Yield (%)
2	***Strychnos colubrina***	Hexane	light yellow	sticky, solid	0.63
		Toluene	Light green,	solid, sticky	4.37
		Chloroform	Sticky,	semisolid, green dark	6.42
		Ethanol	Dark green,	sticky, semisolid	7.02
		Ethanol: Water (1:1)	brick red color	Nonsticky, solid, powder,	9.53

	Strychnos colubrina	Ethanol	Cold maceration	8.98

Table No.10. The percentage yield and physical characteristics of *Boswellia ovalifoliolata* plant leaves extracts.

S. No	Plant	Extract	Appearance	Consistency	Yield (%)
3	***Boswellia ovalifoliolata***	Hexane	Green,	sticky, solid	0.78
		Toluene	Black,	sticky, semisolid	3.83
		Chloroform	buff color,	Solid, Amorphous powder,	5.76
		Ethanol	Brown,	solid, nonsticky	8.97
		Ethanol: Water (1:1)	Brown,	amorphous powder, solid	12.67
	Boswellia ovalifoliolata	Ethanol	Cold maceration		18.90

5.4 Preliminary Phytochemical Screening:

The results of preliminary screening of different plant leaves of *Vitex leucoxylon, Strychnos colubrina,* and *Boswellia ovalifoliolata,* with hexane, toluene, chloroform, ethanol, and ethanol: water (1:1) are given in the table no.17. based on the phytochemical chemical screening ethanolic extract contain alkaloids, flavanoids, tannins and steroids.

Table No.11 Phytochemical Analysis of extracts of *Vitex leucoxylon* leaves:

Method	VL-HE	VL-TE	VL-CHCL3E	VL-OHE	VL-HAE
"Alkaloids"					
"Mayer's test"	+	+	+	+	-
"Wagner's test"	+	+	+	+	+
"Hager's test"	+	+	+	+	+
"Dragendorff's test"	+	+	+	+	+

"Tannic acid test"	-	+	+	+	-
"Flavonoids"					
"Lead acetate test"	+	+	+	+	+
"Shinoda test"	+	-	+	+	-
"Alkaline test"	+	+	+	+	+
"Zinc Hcl test"	+	-	+	+	+
"Tannins"					
"$Fecl_3$ test"	+	-	+	+	+
"Lead acetate test"	+	-	+	+	+
"Phenols"					
"$Fecl_3$ test"	+	-	+	+	+
"Lead acetate test"	+	-	+	+	+
"Steroids& triterpenoids"					
"Libermann-Buchard test"	+(steroids)	-	+(steroids)	+(steroids)	+(triterpenoids)
"Salkowski test"	+(steroids)	-	+(steroids)	+(steroids)	+(steroids)
"Anthocyanins"	-	-	-	-	-
"Glycosides"	+	-	+	+	+
"Saponins"					
"Honeycomb test"	-	-	-	-	-
"Foam test"	-	-	-	-	-
"Anthraquinone"	+	-	-	+	-

VL-HE = *Vitex leucoxylon* Hexane Extract

VL-TE = *Vitex leucoxylon* Toluene Extract

VL-CHCL3E = *Vitex leucoxylon* Chloroform Extract

VL-OHE = *Vitex leucoxylon* alcoholic Extract

VL-HAE = *Vitex leucoxylon* Hydroalcoholic Extract

'+' indicates present

'-' indicates absent

Table No.12. Phytochemical Analysis of extracts of *Strychnos colubrine* leaves:

Method	**SC-HE**	**SC-TE**	**SC-CHCL3E**	**SC-OHE**	**SC-HAE**
Alkaloids					
Mayer's test	+	+	+	+	-
Wagner's test	+	+	+	+	-
Hager's test	+	+	+	+	-
Dragendorff's test	+	+	+	+	-
Tannic acid test	+	+	+	-	-
Flavonoids					
Lead acetate test	+	-	+	+	+
Shinoda test	+	-	-	+	-
Alkaline test	+	-	+	+	+
Zinc Hcl test	+	-	-	+	-
Tannins					
$Fecl_3$ test	+	-	-	+	+
Lead acetate test	+	-	+	+	+
Phenols					
$Fecl_3$ test	+	-	-	+	+
Lead acetate test	+	-	+	+	+
Steroids& triterpenoids					
Libermann-Buchard test	+(triterpenoids)	-	+(triterpenoids)	+(steroids)	+(steroids)
Salkowski test	+(steroids)	-	+(steroids)	+(steroids)	-
Anthocyanins	-	-	-	-	-
Glycosides	+	-	+	+	+
Saponins					
Honeycomb test	+	-	-	+	-
Foam test	+	-	-	+	+
Anthraquinone	-	-	-	+	-

SC-HE = *Strychnos colubrina* Hexane Extract

SC-TE = *Strychnos colubrina* Toluene Extract

SC-CHCL3E = *Strychnos colubrina* Chloroform Extract

SC-OHE = *Strychnos Colubrina* alcoholic Extract

SC-HAE = *Strychnos Colubrina* Hydroalcoholic Extract

'+' indicates present

'-' indicates absent

Table No.13. Phytochemical Analysis of residues of leaves of *Boswellia ovalifoliolata*:

Method	**BO-HE**	**BO-TE**	**BO-CHCL3E**	**BO-OHE**	**BO-HAE**
Alkaloids					
Mayer's test	-	+	-	-	-
Wagner's test	-	+	-	-	-
Hager's test	-	+	-	-	-
Dragendorff's test	-	+	-	-	-
Tannic acid test	-	+	-	-	-
Flavonoids					
Lead acetate test	-	+	-	+	+
Shinoda test	-	+	-	-	-
Alkaline test	-	+	-	+	+
Zinc Hcl test	-	-	-	-	-
Tannins					
$Fecl_3$ test	-	+	-	+	+
Lead acetate test	-	+	-	+	+
Phenols					
$Fecl_3$ test	-	+	-	+	+
Lead acetate test	-	+	-	+	+
Steroids& triterpenoids					
Libermann-Buchard test	-	+(triterpenoids)	-	+(triterpenoids)	+(triterpenoids)
Salkowski test	-	+(steroids)	-	+(steroids)	+(steroids)
Anthocyanins	-	-	-	-	-
Glycosides	-	+	-	+	+
Saponins					

Honeycomb test	-	+	-	+	+
Foam test	-	-	-	+	+
Anthraquinone	-	-	-	-	-

BO-HE = *Boswellia ovalifoliolata* Hexane Extract

BO -TE = *Boswellia ovalifoliolata* Toluene Extract

BO -CHCL3E = *Boswellia ovalifoliolata* Chloroform Extract

BO -OHE = *Boswellia ovalifoliolata* alcoholic Extract

BO -HAE = *Boswellia ovalifoliolata* Hydroalcoholic Extract

'+' indicates present

'-' indicates absent

5.5. CHARACTERIZATION

5.5.1 IR Spectroscopy:

Table. No. 14. IR Spectra of all the extracts of selected medicinal plants

S. No	**Plant residue**	**Wave number cm^{-1}**	**Possible bonds**	**Possible molecule**
1. *Vitex leucoxylon*				
	1.1.VL-H extract	798.75, 1018.61, 1259.31, 1452.54, 1512.01, 1605.58, 1715.17, 2852.97, 2919.31, 2961.65.	C-H bend, CO-O-CO, C-O, C-H bend, N-O, C=C, C=O, C-H, O-H, O-H.	1,2,3-trisubstituted, anhydride, alkyl aryl ether, alkane, nitro compound, conjugated alkene, aliphatic ketone, alkane, alcohol, alcohol.
	1.2.VL-T extract	653.25, 712.26, 804.38, 1028.38, 1262.46, 2368.12, 2963.55,	C-I, C-Br, C=C bend, S=O, C-O, O=C=O, C-H, N-	Halo compound, alkene,

		3311.03, 3407.29, 3706.84.	H 2º amine, N-H 1º amine, O-H.	sulfoxide, alkyl aryl ether, carbon dioxide, alkane, 2º amine, 1º amine,
	1.3.VL-$CHCl_3$ extract	659.42, 729.27, 1023.87, 1093.53, 1461.64, 1592.06, 1724.51, 2198.03, 2359.87, 2916.88, 3362.78, 3511.14, 3704.43.	C-Br, C=C bend, S=O, C-O, C-H bend, N-O, C=O, C=C, O=C=O, C-H, C-H, N-H, O-H.	Halo compound, alkene, sulfoxide, 1º alcohol, alkane, nitro compound, aliphatic ketone, alkene, carbon dioxide, alkane, amine, alcohol.
	1.4.VL-OH extract	772.75, 1027.31, 1605.48, 1694.33, 2856.87, 2923.05, 3327.96.	C=C bend, S=O, C=C, C=O, C-H, C-H, N-H.	Alkene, sulfoxide, alkene, 1º amide, alkane, amine.
	1.5.VL-HA extract	654.00, 808.90, 1266.55, 1582.66, 2343.25, 2800.89, 3216.15, 3674.53.	C-Br, C=C bend, C-O, N-H bend, O=C=O, C-H, C-H, O-H.	Halo compound, alkene, alkyl aryl ether, amine,

				carbon dioxide, aldehyde, alkyne, alcohol.
2. *Strychnos colubrina*				
	2.1. SC-H extract	800.16, 1020.64, 1259.23, 1372.16, 1454.66, 1718.15, 2852.87, 2960.59.	C=C bend, S=O, C-O, S=O, C-H bend, C=O, O-H, C-H.	Alkene, sulfonate, alkyl aryl ether, sulfonamide, alkane, carboxylic acid, alcohol, alkane.
	2.2. SC -T extract	670.50, 2363.26, 3761.51.	C=C bend, O=C=O, O-H.	Alkene, carbon dioxide, alcohol.
	2.3. SC -CHCL3 extract	833.92, 1374.22, 1446.50, 2921.30.	C=C bend, S=O, C-H bend, N-H.	alkene, sulfonate, alkane, amine.
	2.4. SC -OH extract	776.74, 1042.88, 1245.92, 1606.60, 1716.57, 2857.86, 2924.52, 3338.08.	C=C bend, CO-O-CO, C-N, C=C, C=O, O-H, C-H, N-H.	Alkene, anhydride, amine, alkene, carboxylic acid, alcohol, alkane, amine.
	2.5. SC -HA extract	715.18, 1022.38, 1262.54, 3712.23.	C=C bend, S=O, C-O, O-H.	Alkene, sulfonate,

				alkyl aryl ether, alcohol.
3. *Boswellia ovalifoliolata*				
	3.1. BO-H extract	799.32, 1020.49, 1259.71, 1459.63, 1717.80, 2917.44.	C=C bend, S=O, C-O, C-H, C=O, O-H.	Alkene, sulfoxide, carboxylic, alkane, carboxylic, alcohol.
	3.2. BO -T extract	803.29, 1032.14, 1168.52, 1457.99, 1719.71, 2922.74.	C-H bend, S=O, C-O, C-H bend, C=O, O-H.	1,2,3-trisubstituted, sulfoxide, tertiary alcohol, alkane, carboxylic acid, alcohol.
	3.3. BO -CHCL3 extract	693.57, 2329.87.	C-Br, O=C=O.	Halo compound, carbon dioxide.
	3.4. BO -OH extract	1068.85, 2365.04.	C-O, O=C=O.	1º alcohol, carbon dioxide.
	3.5. BO -HA extract	738.75, 1023.94, 1601.04, 2365.50, 2887.49.	C=C, C-N, C=C, O=C=O, C-H.	Alkene, amine, carbon dioxide, alkane.

5.6 Gas Chromatography-Mass Spectroscopy (GC-MS) Analysis of *Vitex leucoxylon*:

GC-MS analysis of *Vitex leucoxylon* leaf ethanolic extract identified twenty-six compounds. Table 15 lists the active compounds with their retention time (RT), concentration (peak area%), molecular formula, similarity, and biological activity. Figure No.14 shows the GC-MS chromatogram of the twenty-six detected compounds. The compounds identified by mass spectroscopy are presented. The GC-MS revealed a total number of components in the ethanol extract. The results revealed that .beta.-D-Glucopyranoside, methyl (22.11%), Phytol (15.77%), Hexadecanoic acid, ethyl ester (10.75%), Ethyl linolenate (8.84%), Benzoic acid, 4-hydroxy (5.66%) and 1-Hexacosanol (5.11%) was found as the 6 major components in the ethanol extract, the eight minor compounds such as Neophytadiene (2.54%), Bicyclo [3.2.0] hept-2-ene, 4-ethoxy-, exo- (2.16%), n-Hexadecanoic acid (3.83%), Linolenic acid (4.37%), Linoleic acid ethyl ester (2.93%), Octadecanoic acid, ethyl ester (3.32%), squalene (2.32%), and 2-Hexadecanoyl glycerol (1.27%).

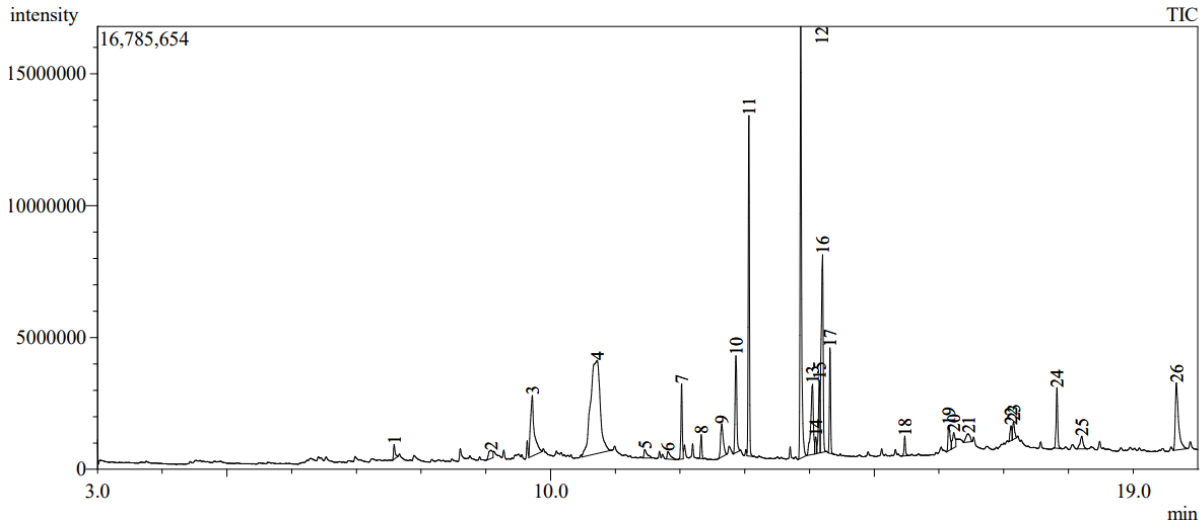


Figure No.14. GC-MS spectra of leaf ethanolic extract of *Vitex leucoxylon*

Table No.15. Bioactive compounds identified in the ethanolic extract of *Vitex leucoxylon*

S. No	Bioactive compound name	Retention time (min)	Peak area %	Molecular formula	Similarity
1	Bicyclo[3.3.0]octan-2-one, 7-ethylidene-	7.583	0.61	$C_{10}H_{14}O$	84
2	Methane, trimethylolnitro-	9.079	0.80	$C_4H_9NO_5$	82
3	Benzoic acid, 4-hydroxy-	9.718	5.66	$C_7H_6O_3$	96
4	beta.-D-Glucopyranoside, methyl	10.721	22.11	$C_7H_{14}O_6$	88
5	Tetradecanoic acid	11.456	0.62	$C_{14}H_{28}O_2$	91
6	2,5-Furandione, 3-(2-decenyl) dihydro-	11.812	0.63	$C_{14}H_{22}O_3$	74
7	Neophytadiene	12.023	2.54	$C_{20}H_{38}$	95
8	Neophytadiene	12.326	0.73	$C_{20}H_{38}$	93

9	Bicyclo [3.2.0] hept-2-ene, 4-ethoxy-, exo-	12.642	2.16	$C_9H_{14}O$	73
10	n-Hexadecanoic acid	12.864	3.83	$C_{16}H_{32}O_2$	94
11	Hexadecanoic acid, ethyl ester	13.063	10.75	$C_{18}H_{36}O_2$	94
12	Phytol	13.865	15.77	$C_{20}H_{40}O$	97
13	Linolenic acid	14.044	4.37	$C_{18}H_{30}O_2$	96
14	3,7,11,15-Tetramethylhexadec-2-en-1-yl acetate	14.097	0.63	$C_{22}H_{42}O_2$	92
15	Linoleic acid ethyl ester	14.150	2.93	$C_{20}H_{36}O_2$	89
16	Ethyl linolenate	14.199	8.84	$C_{20}H_{34}O_2$	95
17	Octadecanoic acid, ethyl ester	14.317	3.32	$C_{20}H_{40}O_2$	94
18	Eicosanoic acid, ethyl ester	15.470	0.68	$C_{22}H_{44}O_2$	92
19	2-Hexadecanoyl glycerol	16.152	1.27	$C_{19}H_{38}O_4$	93
20	Octacosanal	16.230	1.00	$C_{28}H_{56}O$	79
21	Arbutin	16.455	0.84	$C_{12}H_{16}O_7$	91
22	1-cis-Vaccenoylglycerol	17.111	0.68	$C_{21}H_{40}O_4$	85
23	Methyl linolenate	17.152	0.84	$C_{19}H_{32}O_2$	90
24	Squalene	17.821	2.32	$C_{30}H_{50}$	96
25	1-Pentacosanol	18.203	0.95	$C_{25}H_{52}O$	90
26	1-Hexacosanol	19.668	5.11	$C_{26}H_{54}O$	96

Gas chromatography- Mass spectroscopy (GC-MS) analysis of *Strychnos colubrina*

Eighteen compounds have been detected by GC-MS analysis in the studies on the active constituents in the plant *Strychnos colubrina* leaf ethanolic extract. The active compounds with their retention time (RT), molecular formula, molecular weight (MW), and concentration (peak area%) are presented in Table No.22. The GC-MS chromatogram of the eighteen peaks of the compounds detected is shown in Figure no.66. The compounds which the mass spectroscopy identified were presented. The GC-MS revealed a total number of components in the ethanol extract. The results revealed that Phytol (19.72 %), Lupeol (20.06%), Squalene (11.58%) and gamma.-Sitosterol (13.22%) was found as the 4 major components in the ethanol extract, the seven minor compounds such as n-Hexadecanoic acid (5.84%) Hexadecanoic acid, ethyl ester (5.00%), Ethyl .alpha.-linolenate (5.37%), alpha.-Linolenic acid (2.26%), Stigmasterol (4.40%), and beta.-Amyrone (2.76%).

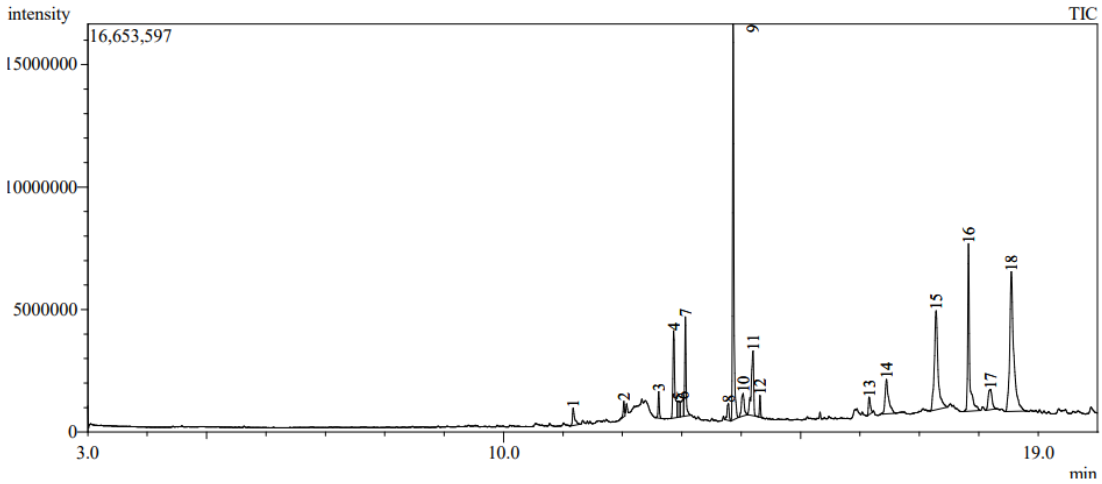


Figure No.15. GC-MS spectra of leaf ethanolic extract of *Strychnos colubrina*

Table No.16. Bioactive compounds identified in the ethanolic extract of *Strychnos colubrina*

S. No	Bioactive compound name	Retention time (min)	Peak area %	Molecular formula	Similarity
1	1,2-O-Isopropylidene-D-xylofuranose, TBDMS derivative	11.170	1.47	$C_{14}H_{28}O_5Si$	72
2	Neophytadiene	12.023	0.77	$C_{20}H_{38}$	95
3	Hexadecanoic acid, methyl ester	12.612	1.22	$C_{17}H_{34}O_2$	96
4	n-Hexadecanoic acid	12.865	5.84	$C_{16}H_{32}O_2$	94
5	Benzoic acid, 4-methyl-2-trimethylsilyloxy-, trimethylsilyl ester	12.944	1.29	$C_{14}H_{24}O_3Si_2$	55
6	trans-2-undecenoic acid	13.020	1.73	$C_{11}H_{20}O_2$	76
7	Hexadecanoic acid, ethyl ester	13.061	5.00	$C_{18}H_{36}O_2$	94
8	Linolenic acid, methyl ester	13.784	1.24	$C_{19}H_{32}O_2$	92
9	Phytol	13.867	19.72	$C_{20}H_{40}O$	97
10	alpha. -Linolenic acid	14.034	2.26	$C_{18}H_{30}O_2$	91
11	Ethyl. alpha. -linolenate	14.198	5.37	$C_{20}H_{34}O_2$	96
12	Octadecanoic acid, ethyl ester	14.317	0.90	$C_{20}H_{40}O_2$	94

13	Glycerol. beta. - palmitate	16.155	1.16	$C_{19}H_{38}O_4$	93
14	Stigmasterol	16.447	4.40	$C_{29}H_{48}O$	85
15	gamma. -Sitosterol	17.280	13.22	$C_{29}H_{50}O$	80
16	Squalene	17.826	11.58	$C_{30}H_{50}$	95
17	beta. -Amyrone	18.194	2.76	$C_{30}H_{48}O$	84
18	Lupeol	18.547	20.06	$C_{30}H_{50}O$	91

5.7 Gas Chromatography-Mass Spectroscopy (GC-MS) Analysis of *Boswellia ovalifoliolata*

Twenty-eight compounds have been detected by GC-MS analysis in the studies on the active constituents in the plant *Boswellia ovalifoliolata* leaf ethanolic extract. The active compounds with their retention time (RT), molecular formula, molecular weight (MW), and concentration (peak area%) are presented in Table-23. The GC-MS chromatogram of the twenty-eight peaks of the compounds detected is shown in Figure-16. The compounds which the mass spectroscopy identified were presented. The GC-MS revealed a total number of components in the ethanol extract. The results revealed that 1,2,3-Benzenetriol (36.16%), Quinic acid (21.84%), and Lupeol (11.75%) was found as the 3 major components in the ethanol extract, the eight minor compounds such as Levoglucosan (3.53%), Hexadecanoic acid, methyl ester (1.59%), n-Hexadecanoic acid (3.29%), Hexadecanoic acid, ethyl ester (1.64%), Methyl linolenate (1.30%), 9,12,15-Octadecatrienoic acid, (Z,Z,Z)- (1.85%), Ethyl .alpha.-linolenate (1.16%), and .gamma.-Sitosterol (1.98%).

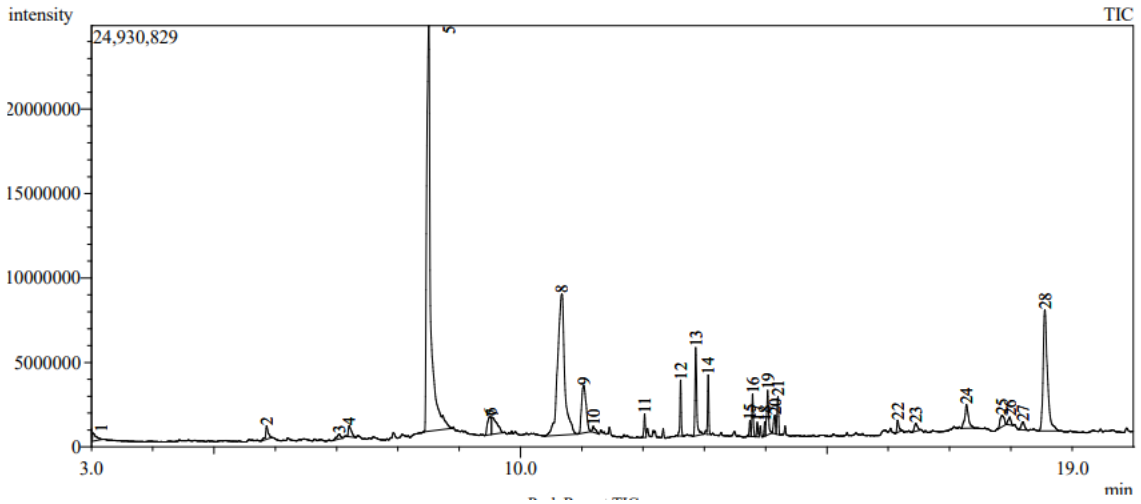


Figure No.16. GC-MS spectra of leaf ethanolic extract of *Boswellia ovalifoliolata*

Table No.17. Bioactive compounds identified in the ethanolic extract of *Boswellia ovalifoliolata*

S. No	Bioactive compound name	Retention time (min)	Peak area %	Molecular formula	Similarity
1	Furfural	3.025	0.64	$C_5H_4O_2$	81
2	Levoglucosenone	9.079	0.83	$C_6H_6O_3$	95
3	1,4:3,6-Dianhydro-.alpha.-d-glucopyranose	7.041	0.43	$C_6H_8O_4$	82
4	5-Hydroxymethylfurfural	7.206	0.74	$C_6H_6O_3$	91
5	1,2,3-Benzenetriol	8.503	36.16	$C_6H_6O_3$	96
6	Levoglucosan	9.505	1.50	$C_6H_{10}O_5$	93
7	Levoglucosan	9.535	2.03	$C_6H_{10}O_5$	87
8	Quinic acid	10.675	21.84	$C_7H_{12}O_6$	83
9	Quinic acid	11.033	4.83	$C_7H_{12}O_6$	81
10	Methyl-3,4-di-O-methylxyloside	11.188	0.39	$C_8H_{16}O_5$	76
11	Neophytadiene	12.024	0.74	$C_{20}H_{38}$	93
12	Hexadecanoic acid, methyl ester	12.613	1.59	$C_{17}H_{34}O_2$	97
13	n-Hexadecanoic acid	12.860	3.29	$C_{16}H_{32}O_2$	93
14	Hexadecanoic acid, ethyl ester	13.062	1.64	$C_{18}H_{36}O_2$	94
15	Methyl linoleate	13.741	0.42	$C_{19}H_{34}O_2$	93
16	Methyl linolenate	13.788	1.30	$C_{19}H_{32}O_2$	95
17	Phytol	13.863	0.46	$C_{20}H_{40}O_2$	98
18	10(E),12(Z)-Conjugated linoleic acid	13.985	0.41	$C_{18}H_{32}O_2$	92
19	9,12,15-Octadecatrienoic acid, (Z, Z,Z)-	14.034	1.85	$C_{18}H_{30}O_2$	96
20	trans, trans-9,12-Octadecadienoic acid, propyl ester	14.148	0.84	$C_{21}H_{38}O_2$	90

21	Ethyl. alpha. -linolenate	14.199	1.16	$C_{20}H_{34}O_2$	94
22	2-Hexadecanoyl glycerol	16.153	0.52	$C_{19}H_{38}O_4$	93
23	Stigmasterol	16.451	0.55	$C_{29}H_{48}O$	85
24	gamma. -Sitosterol	17.274	1.98	$C_{29}H_{50}O$	80
25	beta. -Amyrin	17.855	1.06	$C_{29}H_{50}O$	95
26	Germanicol	17.980	0.49	$C_{30}H_{50}O$	90
27	(1R,2S,8R,8Ar)-8-acetoxy-1-(2-hydroxyethyl)-1,2,5,5-tetramethyl-trans-decalin	18.197	0.58	$C_{16}H_{30}O_2$	71
28	Lupeol	18.555	11.75	$C_{30}H_{50}$	94

5.8 HPTLC, HPLC, Mass spectra and NMR spectra of *Vitex leucoxylon:*

5.8.1 HPTLC chromatogram of polarity based different extracts of *Vitex leucoxylon*

Sample of different extracts of *Vitex leucoxylon* applied on alumina silica plate and run in the solvent system of toulene : ethyl acetate (8:2) and visualized the chromatogram under UV cabinet at 254nm, 366nm and white R. HPTLC analysis of different extracts of *Vitex leucoxylon* were performed and showed in figure.no. 114. Calculated the R_f values of the hexane, chloroform, toluene, ethanol, hydro alcoholic (0.1, 0.11, 0.1, 0.2, 0.09) at 254nm, (0.25, 0.26, 0.28, 0.285) at 366nm and (0.6, 0.61, 0.63) at white R.

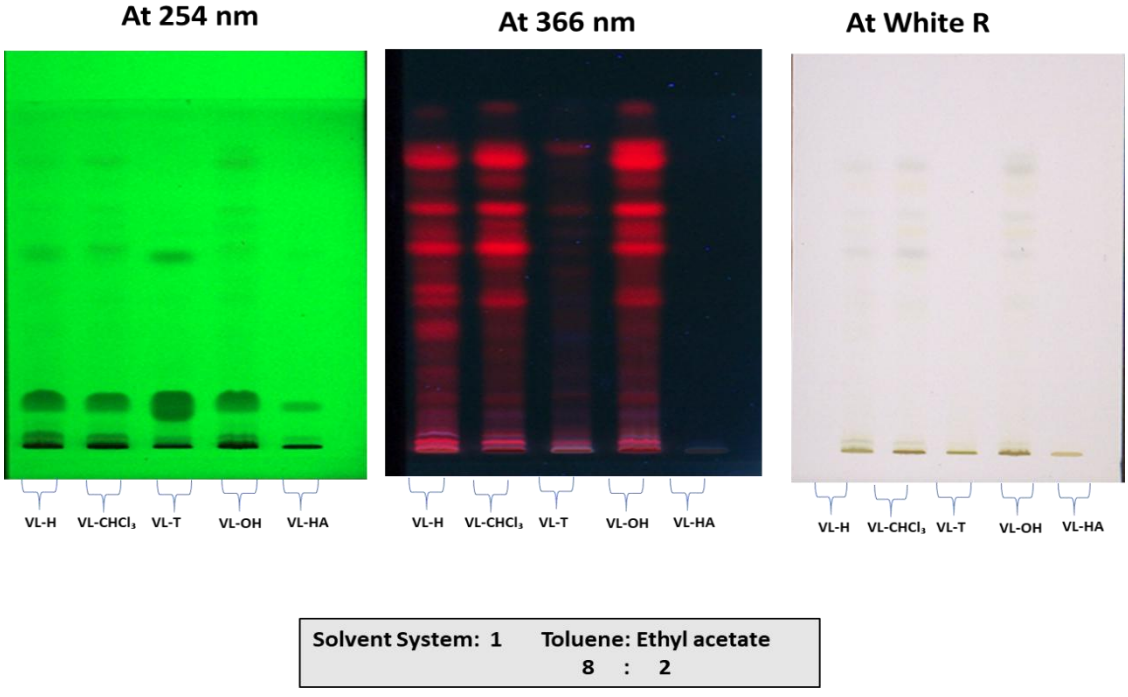


Figure No.17. HPTLC chromatogram of polarity based different extracts of *Vitex leucoxylon*

5.8.2 HPTLC chromatogram of ethanol extract and fractions of *Vitex leucoxylon*

Sample of ethanol extract and fractions of *Vitex leucoxylon* applied on alumina silica plate and run in the solvent system of hexane : ethyl acetate (9:1) and visualized the chromatogram under UV cabinet at 254nm and 366nm. HPTLC chromatogram of ethanol extract and fractions of *Vitex leucoxylon* shown in figure no.115. Crude ethanolic and fractions are shown R_f values 0.08, 0.1, fraction 2 (0.21), fraction 3 (0.24), fraction 8 (0.8, 0.1, 0.18), fraction 9 (0.08) and fraction 10 (0.075) at 254nm.

Crude ethanolic and fractions are shown R_f values 0.08, 0.1, fraction 2 (0.21), fraction 3 (0.24), fraction 8 (0.8, 0.1, 0.18), fraction 9 (0.08) and fraction 10 (0.075) at 366nm.

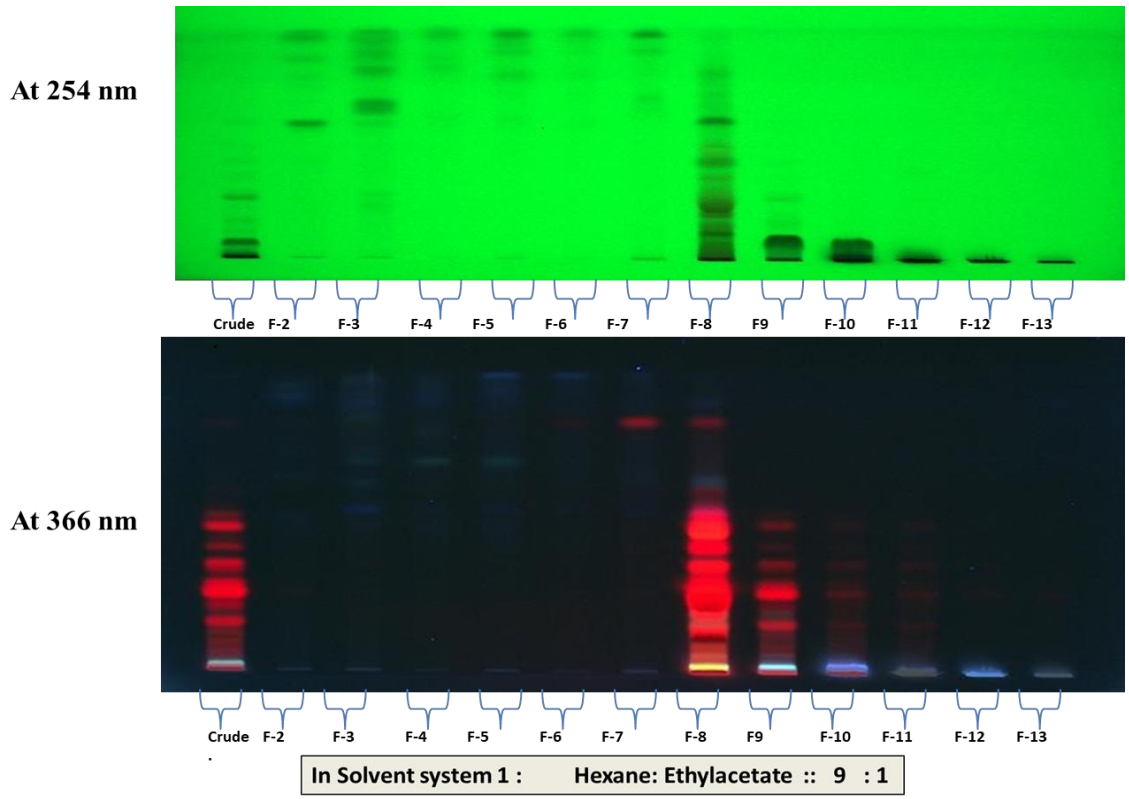

At 254 nm
Crude F-2 F-3 F-4 F-5 F-6 F-7 F-8 F9 F-10 F-11 F-12 F-13
At 366 nm
Crude F-2 F-3 F-4 F-5 F-6 F-7 F-8 F9 F-10 F-11 F-12 F-13
In Solvent system 1 : Hexane: Ethylacetate :: 9 : 1

Figure No.18. HPTLC chromatogram of ethanol extract and fractions of *Vitex leucoxylon*

5.8.3 HPTLC chromatogram of ethanol extract and fractions of *Vitex leucoxylon*

Sample of Inugalactolipid-A applied on alumina silica plate and run in the solvent system of toluene : ethyl acetate : methanol (7:2:1) and visualized the chromatogram under UV cabinet at 254nm and 366nm. HPTLC Chromatogram of Inugalactolipid-A shown in figure no.19. R_f values of VL crude is 0.13 and VL IGL is 0.12 at 254nm and 0.09, 0.088 at 366nm.

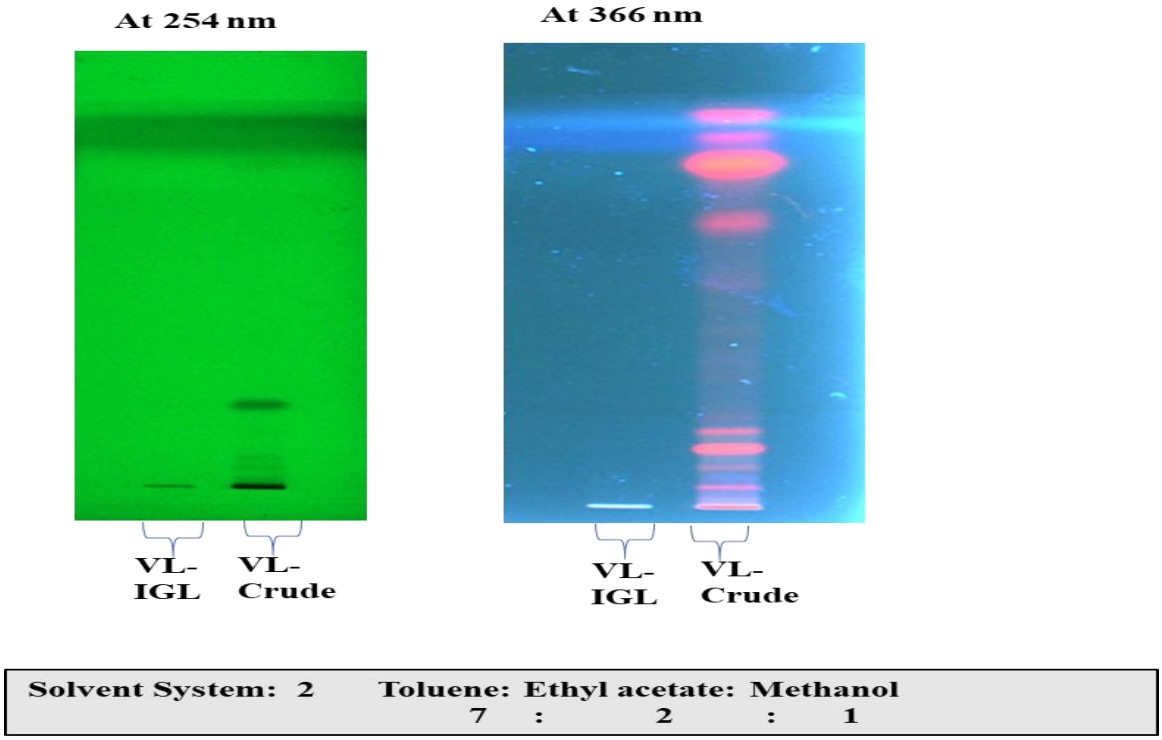


Figure No.19. HPTLC Chromatogram of Inugalactolipid-A

5.8.4 HPLC chromatogram of Inugalactolipid-A:

The chromatogram peak of Inugalactolipid-A was eluted at 14.396 min at 254nm with PDA detector and the chromatogram was shown in figure no.20.

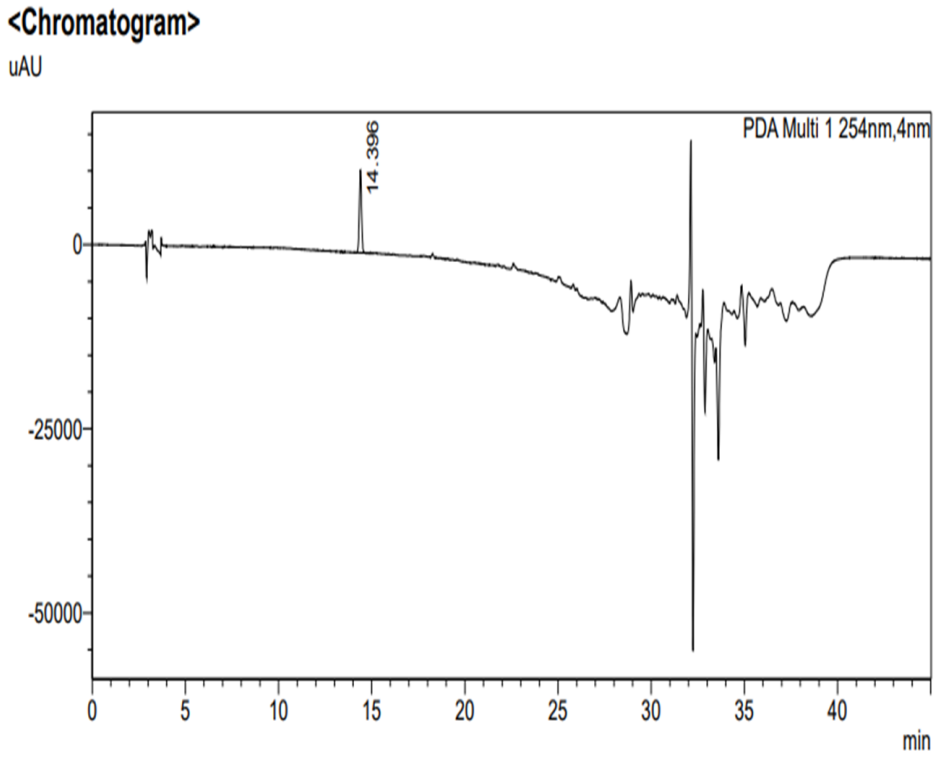
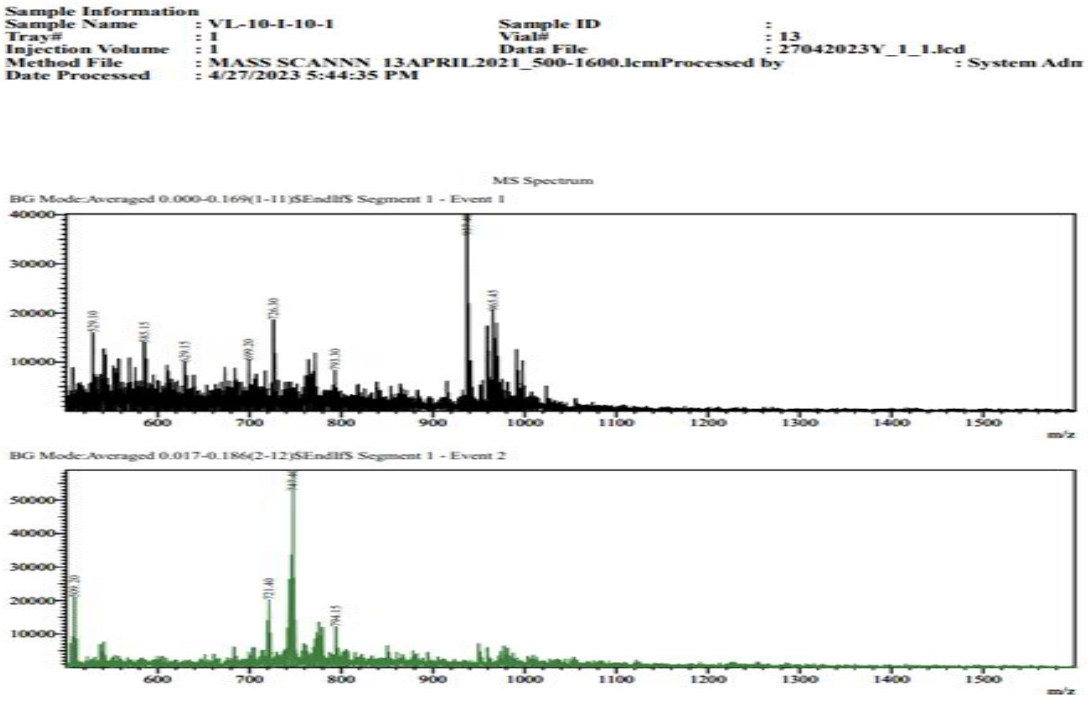


Figure No.20. HPLC chromatogram of Inugalactolipid-A

5.8.5 Mass spectra of Inugalactolipid-A

The ES-MS spectrum of Inugalactolipid-A (figure no.21) showed a major peak at m/z (-ev) 915.10 with minor peaks at 963.45. MS/MS analysis on the major ion, m/z (+ev) 915.10.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Sample Name : VL-10-1-10-1
Sample ID :
Tray# : 1
Vial# : 13
Injection Volume : 1
Data File : 27042023Y_1_1.lcd
Method File : MASS SCANNN_13APRIL2021_500-1600.lcmProcessed by : System Adn
Date Processed : 4/27/2023 5:44:35 PM
MS Spectrum
BG Mode:Averaged 0.000-0.169(1-11)|SEndIfS Segment 1 - Event 1
BG Mode:Averaged 0.017-0.186(2-12)|SEndIfS Segment 1 - Event 2
m/z

Figure No.21. Mass spectra of Inugalactolipid-A

5.8.6 NMR spectra of Inugalactolipid-A:

Recorded NMR spectra of Inugalactolipid-A was shown in figure no.22.

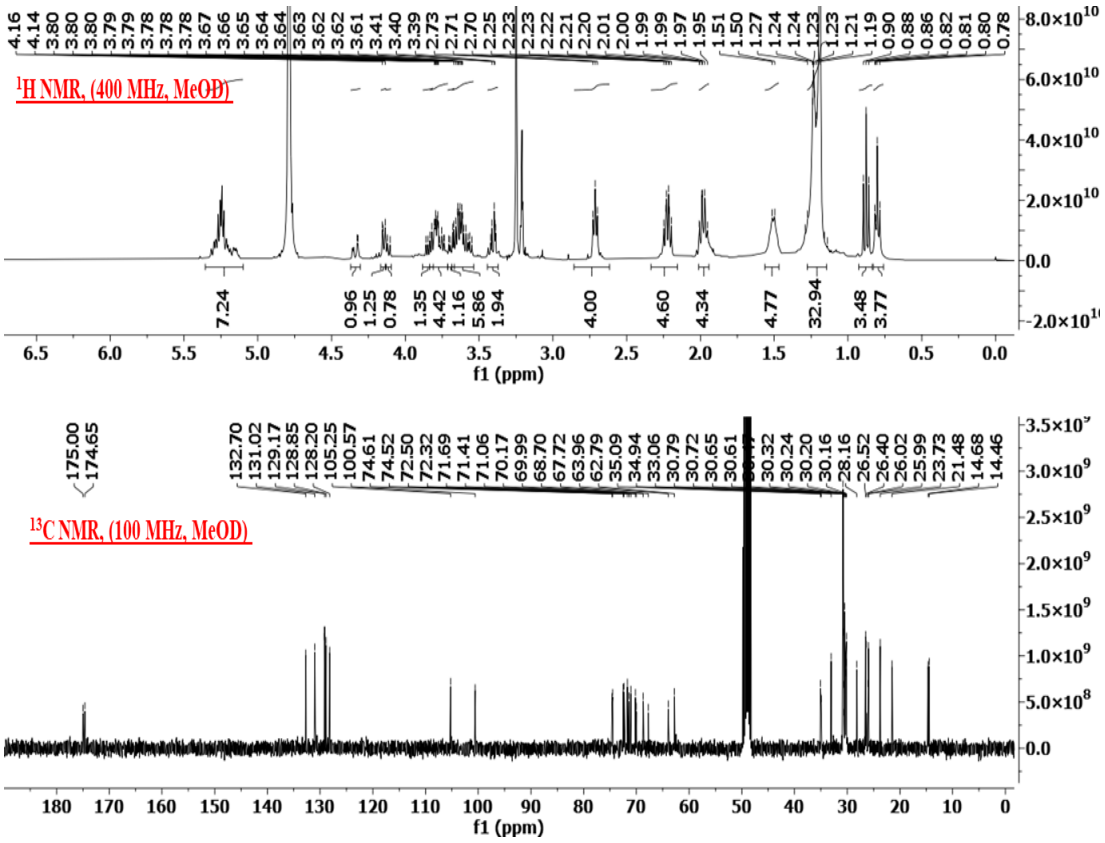

Figure No.22. NMR spectra of Inugalactolipid-A

5.8.7 ^{1}H and ^{13}C NMR data of Inugalactolipid-A as following:

Inugalactolipid-A: ^{1}H NMR (400 MHz, MeOD) δ 7.20 (s, 2H), 6.28 (d, J = 2.1 Hz, 1H), 6.10 (d, J = 2.1 Hz, 1H), 5.15 (d, J = 7.6 Hz, 1H), 3.63 (dd, J = 11.9, 2.4 Hz, 1H), 3.55 – 3.49 (m, 1H), 3.42 (dd, J = 8.8, 7.7 Hz, 2H), 3.35 (t, J = 8.8 Hz, 1H), 3.32 – 3.26 (m, 1H), 3.14 (ddd, J = 9.4, 5.1, 2.3 Hz, 1H)

^{13}C NMR (101 MHz, MeOD) δ 178.03, 164.62, 161.61, 157.61, 157.00, 145.02, 136.67,

5.8.8 Structure of Inugalactolipid-A:

Inugalactolipid-A was isolated from *Vitex leucoxylon* and isolated compound of Inugalactolipid-A was shown in figure no.23.

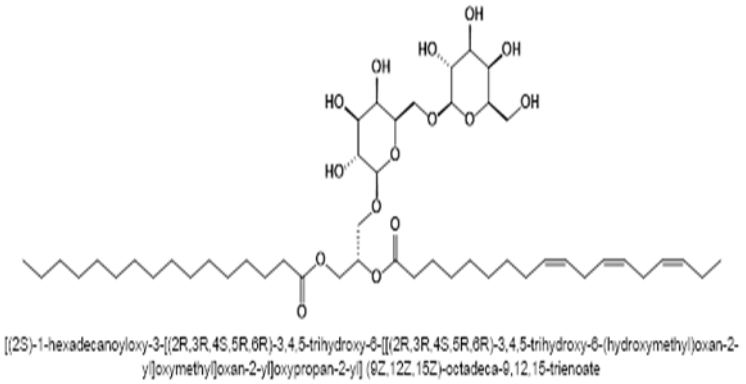

Figure No.23. Structure of Inugalactolipid-A

5.8.9 Isolation of Inugalactolipid-A from *Vitex leucoxylon* plant:

Isolated compound of Inugalactolipid-A from *Vitex leucoxylon* was shown in figure no.24.

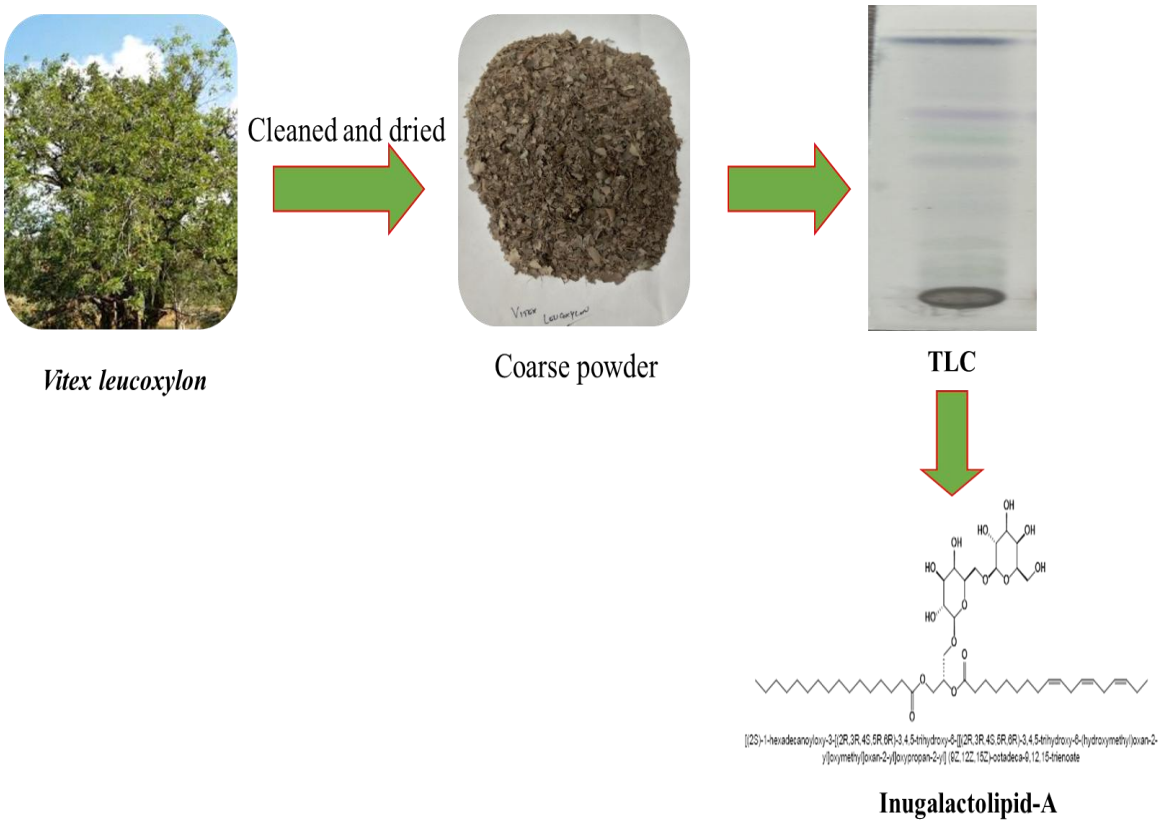

Figure No: 24. Isolation of Inugalactolipid-A from *Vitex leucoxylon* plant

5.9 HPTLC, HPLC, Mass spectra and NMR spectra of *Strychnos colubrina*

5.9.1 HPTLC chromatogram of polarity based different extracts of *Strychnos colubrina*

Sample of different extracts of *Strychnos colubrina* applied on alumina silica plate and run in the solvent system of toulene : ethyl acetate (8:2) and visualized the chromatogram under UV cabinet at 254nm, 366nm and white R. HPTLC analysis of different extracts of *Strychnos colubrina* were performed and showed in figure.no. 122. Calculated the R_f values of the toluene, ethanol, hydroalcoholic (0.8, 0.81, 0.81) at 254nm, ethanol (0.82, 0.821, 0.84) at 366nm and (0.7) ethanol (0.82, 0.821, 0.84) at white R.

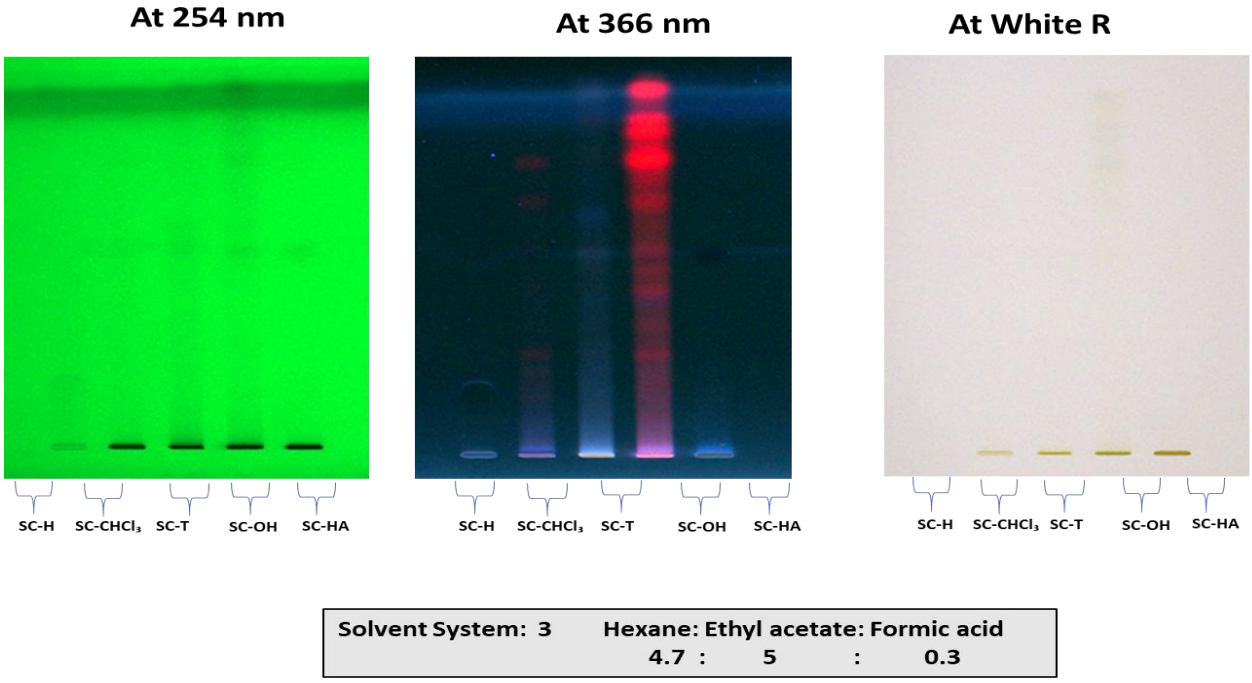

Figure No.25. HPTLC chromatogram of polarity based different extracts of *Strychnos colubrina*

5.9.2 HPLC chromatogram of oleanolic acid from *Strychnos colubrina*

The HPLC chromatogram peak of oleanolic acid was eluted at 6.285 min at 207nm with PDA detector and the chromatogram was shown in figure no.26.

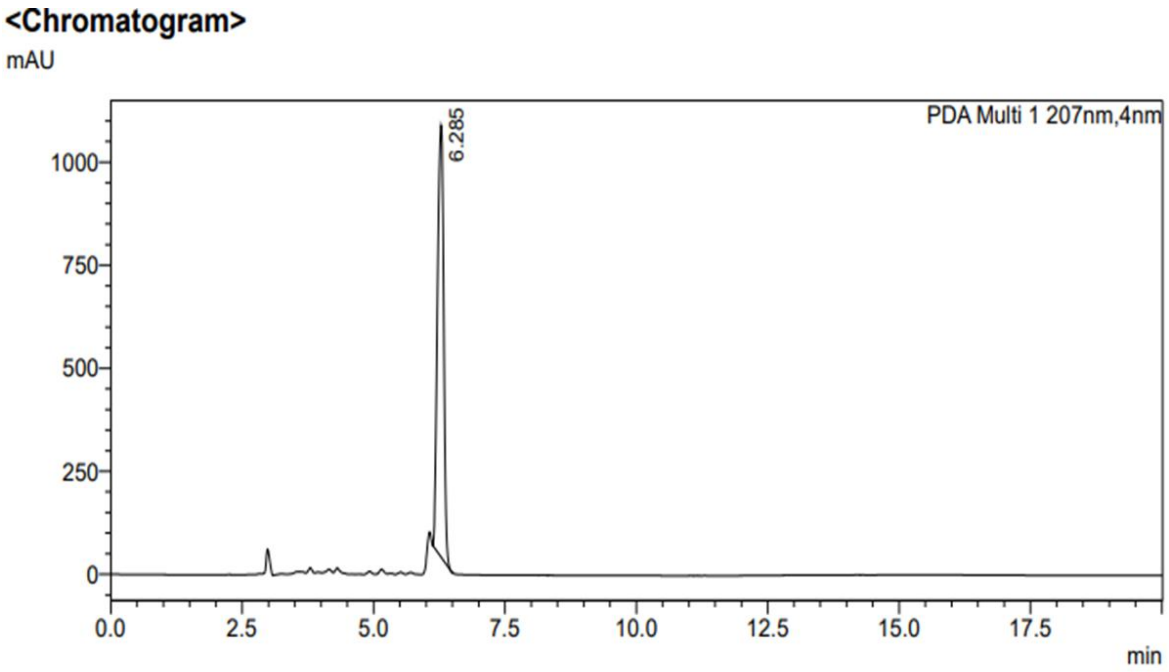

Figure No.26. HPLC chromatogram of oleanolic acid from *Strychnos colubrina*

5.9.3 HPLC chromatogram of ursolic acid from *Strychnos colubrina*

The HPLC chromatogram peak of Ursolic was eluted at 6.079 min at 207nm with PDA detector and the chromatogram was shown in figure no.27.

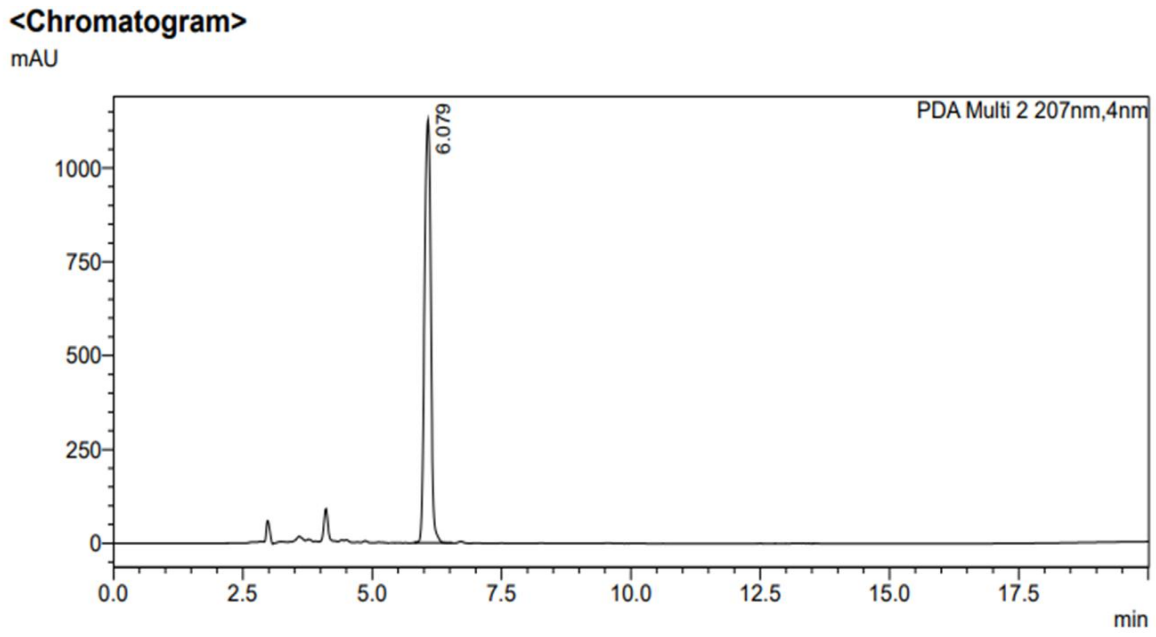

Figure No.27. HPLC chromatogram of ursolic acid from *Strychnos colubrina*

5.9.3 HPLC chromatogram of lupeol acid from *Strychnos colubrina*

The HPLC chromatogram peak of lupeol was eluted at 3.933 min at 207nm with PDA detector and the chromatogram was shown in figure no.28.

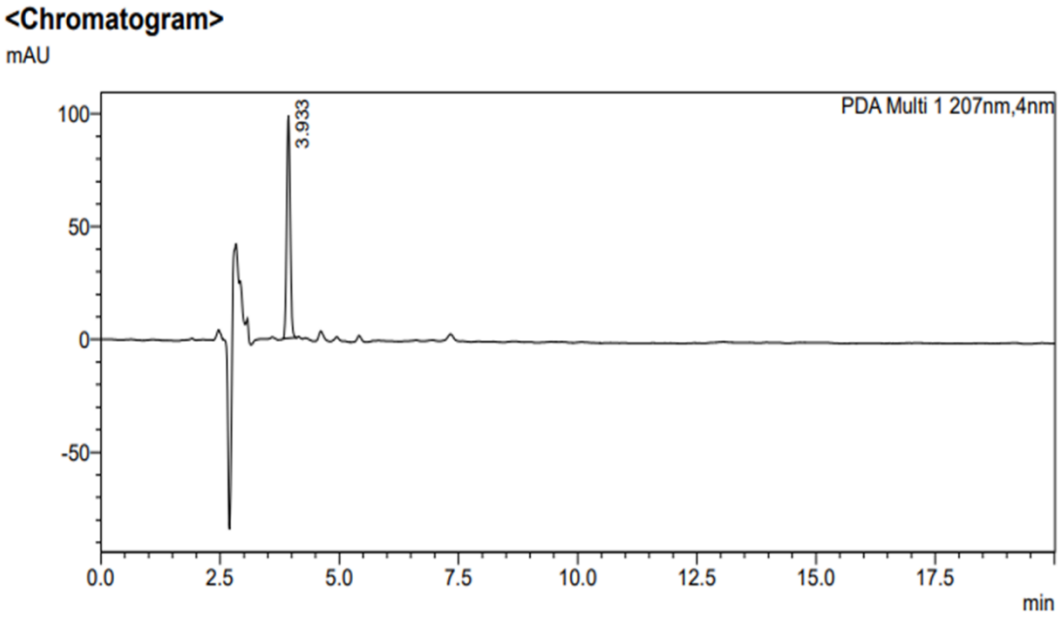

Figure No.28. HPLC chromatogram of lupeol acid from *Strychnos colubrina*

5.9.4 HPLC chromatogram of cirsimarin acid from *Strychnos colubrina*

The HPLC chromatogram peak of cirsimarin was eluted at 24.743, 24.744 min at 254nm and 366nm with PDA detector and the chromatogram was shown in figure no.29.

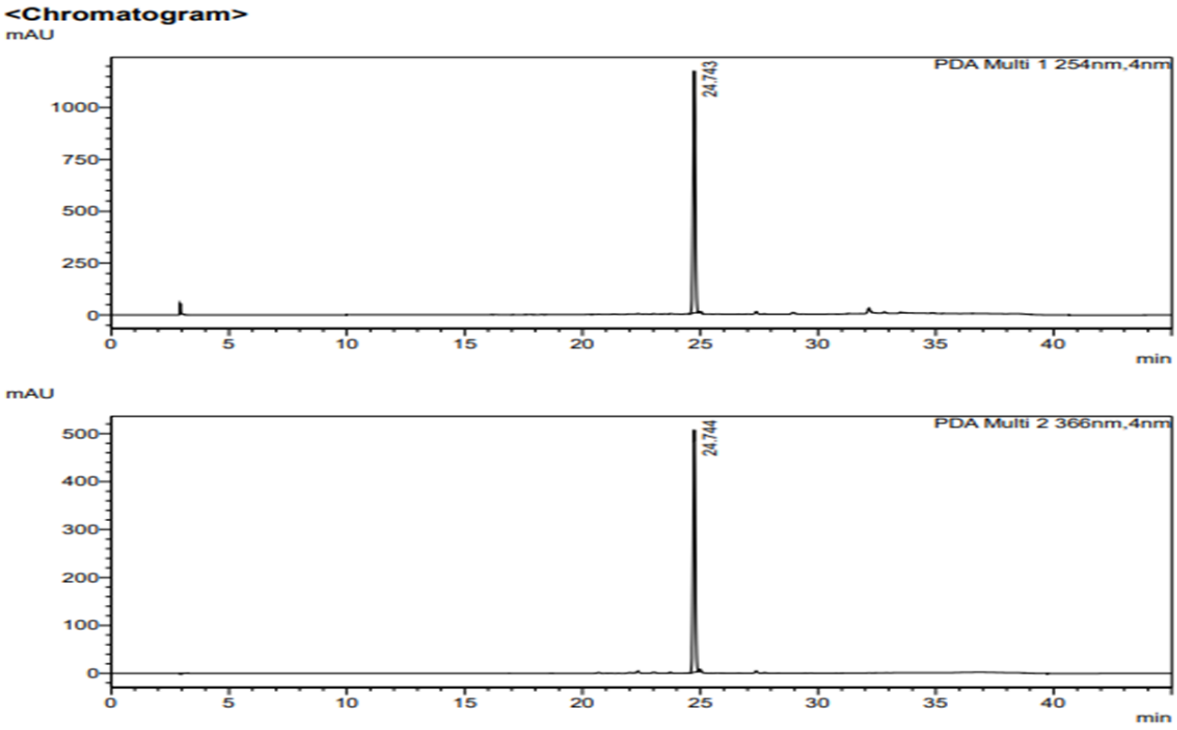

Figure No.29. HPLC chromatogram of cirsimarin acid from *Strychnos colubrina*

5.9.5 HRMS spectra of cirsimarin from *Strychnos colubrina*

The ES-MS spectrum of cirsimarin (figure no.30) showed a major peak at m/z (-ev) 477.1393 with minor peaks at 478.1429. MS/MS analysis on the major ion, m/z (+ev) 477.1393.

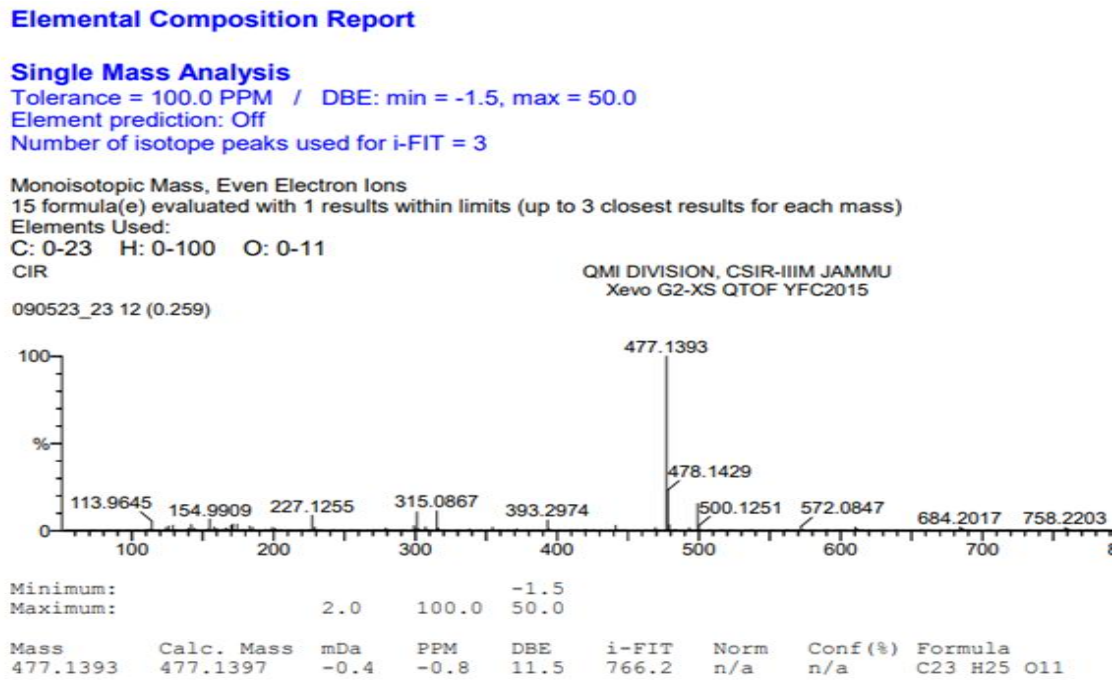

Figure No.30. HRMS spectra of cirsimarin from *Strychnos colubrina*

5.9.6 NMR spectra of cirsimarin from *Strychnos colubrina:*

Recorded NMR spectra of cirsimarin was shown in figure no.31.

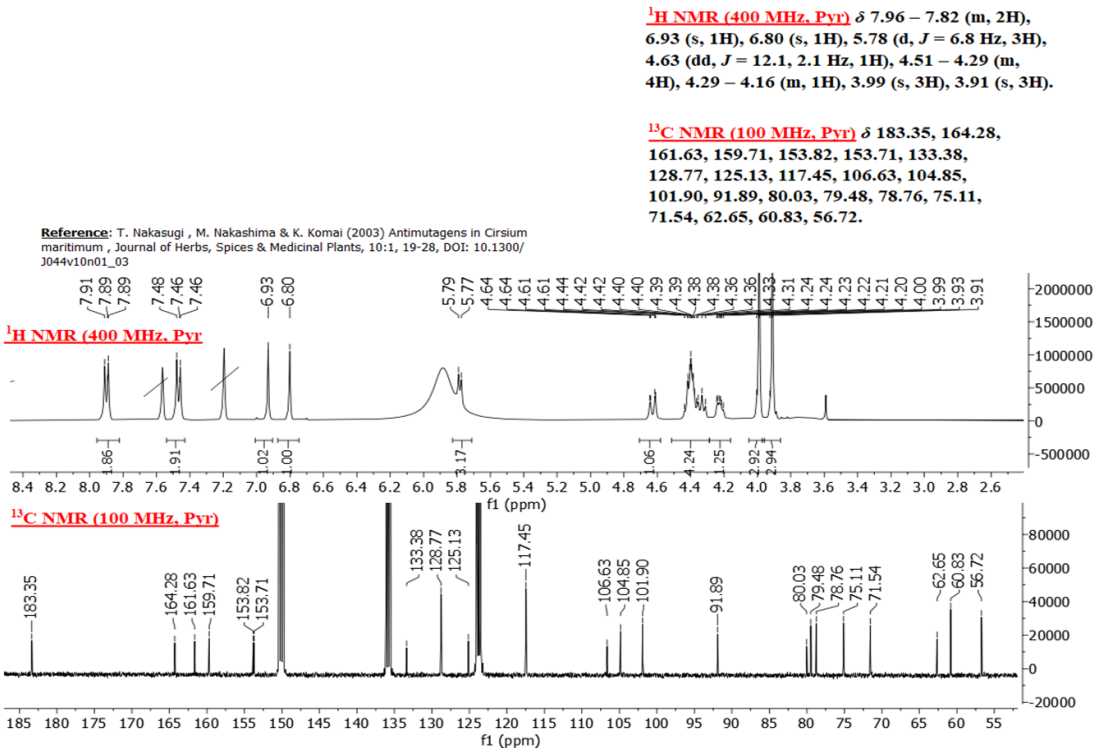

Figure No.31. NMR spectra of cirsimarin from *Strychnos colubrina*

5.9.7 ^{1}H and ^{13}C NMR data of Cirsimarin, Oleanolic acid, Lupeol, β-sitosterol, Ursolic acid as following:

Cirsimarin: ^{1}H NMR (400 MHz, Pyr) δ 7.96-7.82 (m, 2H), 6.93 (s, 1H), 5.78 (d, *J*= 6.8 Hz, 3H), 4.63 (dd, *J*= 12.1, 2.1 Hz, 1H), 4.51-4.29 (m, 4H), 4.29- 4.16 (m, 1H), 3.99 (s, 3H), 3.91 (s, 3H); ^{13}C NMR (100 MHz, Pyr) δ 183.35, 164.28, 161.63, 159.71, 153.82, 153.71, 133.38, 101.90, 91.89, 80.03, 79.48, 78.76, 75.11, 71.54, 62.65, 60.83, 56.72 ($C_{23}H_{24}O_{11}$, m/z 477.1393).

Oleanolic acid: ^{1}H NMR (500 MHz, C_5D_5N) δ 5.48 (t, J = 3.3 Hz, 1H), 3.42 (dd, J = 10.6, 5.5 Hz, 1H), 3.29 (dd, J = 13.8, 4.0 Hz, 1H), 2.07-2.20 (m, 2H), 2.00-2.06 (m, 1H), 1.93-1.96 (m, 3H), 1.78-1.83 (m, 4H), 1.68 (t, J = 8.9 Hz, 1H), 0.85-1.58 (m, 11H), 1.27 (s, 3H), 1.22 (s, 3H), 1.02 (s, 3H), 1.01 (s, 3H), 1.00 (s, 3H), 0.94 (s, 3H), 0.89 (s, 3H); ^{13}C NMR (125 MHz, C5D5N) δ 181.4, 146.1, 123.8, 79.4, 57.1, 49.4, 48.0, 47.8, 43.5, 43.3, 41.1, 40.6, 40.2, 38.7, 35.5, 34.6,

34.5, 34.5, 32.2, 30.0, 29.6, 29.4, 27.4, 25.1, 25.1, 25.0, 20.1, 18.7, 17.8, 16.8 ($C_{30}H_{48}O_3$, m/z 456.70).

Lupeol: White powder; Molecular formula: $C_{30}H_{50}O$; Exact mass: 426.72; SC-15; ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 4.69 (1H, *s*, H-29a), 4.56 (1H, *s*, H-29b), 3.19 (1H, *m*, H-3), 2.38 (1H, *td*, *J* = 11.0, 5.8 Hz, H-19), 1.92 (1H, *m*, H-1), 1.68 (3H, *s*, H-30), 1.38 (2H, *m*, H-6a, b), 1.35 (1H, *m*, H-18), 1.22 (1H, *m*, H-9), 1.03 (3H, *s*, H-26), 0.97 (3H, *s*, H-23), 0.94 (3H, *s*, H-27), 0.83 (3H *s*, H-25), 0.79 (3H, *s*, H-23), 0.76 (3H, *s*, H-28), 0.68 (1H, *m*, H-5); ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 151.30 (C-20), 109.63 (C-29), 79.30 (C-3), 55.57 (C-5), 50.71 (C-9), 48.58 (C-18), 48.28 (C-19), 43.20 (C-17), 43.15 (C-14), 41.11 (C-8), 40.29 (C-22), 39.15 (C-4), 38.98 (C-1), 38.32 (C-13), 37.45 (C-10), 35.87 (C-16), 34.55 (C-7), 30.13 (C-21), 28.28 (C-23), 27.71 (C-2), 27.70 (C-15), 25.40 (C-12), 21.21 (C-11), 19.60 (C-30), 18.61 (C-6), 18.30 (C-28), 16.42 (C-25), 16.26 (C-26), 15.67 (C-24), 14.84 (C-27). HR-ESIMS *m/z* 449.3722 $[M+Na]^+$ (calcd for $C_{30}H_{50}ONa$, m/z 449.3715).

β-sitosterol: White powder, ^{1}H NMR (400 MHz, $CDCl_3$): δ H 5.34 (1H, m, H-11), 3.52 (1H, m, H-18), 2.28 (2H, m, H-17), 1.99 (2H, m, H-10, H-6), 1.85 (2H, m, H-14, H-15), 1.52 (3H, s, Me-19), 1.53 (2H, m, H-28, H-24), (1H, m, H-24), 1.00 (3H, d, Me-21), 1.25 (1H, d, H-9), 0.91 (3H, t, Me-29), 0.84 (3H, d, Me-26), 0.80 (3H, d, Me-27), 0.68 (3H, s, Me-18), ^{13}CNMR (100 MHz, CDCl3); δ C 140.9 (C-5), 121.8 (C-6), 71.8 (C-3), 57.0 (C-14), 56.9 (C-17), 50.3 (C-9), 45.9 (C-22), 42.5 (C-4), 42.3 (C-13), 39.9 (C-12), 37.3 (C-1), 36.3 (C-10), 36.1 (C-18), 33.8 (C-20), 32.0 (C-7), 32.00 (C-8), 31.7 (C-2), 29.8 (C-25), 28.4 (C-16), 26.2 (C-15), 25.5 (C-21), 24.4 (C-23), 21.2 (C-11), 20.3 (C-26), 19.5 (C-27), 19.1 (C-19), 18.9 (C-28), 12.1 (C-29), 11.9 (C-24). ESIMS m/z 415.71$[M+H]^+$ (cal for $C_{29}H_{50}O$, m/z 414.71).

Ursolic acid: White powder, ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 11.17 (s, 1H), 8.89 (s, 1H), 6.85 (d, *J* = 10.0 Hz, 1H), 5.85 (d, *J* = 11.3 Hz, 1H), 5.72 – 5.62 (m, 2H), 5.59 – 5.54 (m, 3H), 5.33 (dd, *J* = 24.2, 12.4 Hz, 6H), 5.19 (d, *J* = 46.8 Hz, 8H), 5.00 (s, 5H), 4.92 (s, 1H), 4.86 (t, *J* = 7.0 Hz, 1H), 4.75 (s, 4H), 4.63 (s, 4H), 4.61 (s, 3H), 4.59 (s, 4H), 4.52 (d, *J* = 6.4 Hz, 4H), 4.48 (s, 4H), 4.43 (s, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ c 184.30, 142.13, 129.44, 82.29, 59.27, 56.85, 52.62, 45.82, 43.32, 42.97 42.92, 42.58, 42.44, 40.68, 36.89, 34.39, 31.79, 31.46, 30.44, 27.92, 26.98, 26.88, 24.34, 22.08, 20.43, 20.37, 19.08, 18.76. ESIMS m/z 456.75 $[M+H]^+$ (cal for $C_{30}H_{48}O_2$, m/z 457.75).

5.9.8 Isolation of cirsimarin, beta sitosterol, lupeol, Ursolic acid, and olenolic acid from *Strychnos colubrina* plant

Isolated molecules of cirsimarin, beta sitosterol, lupeol, ursolic acid, and olenolic acid from *Strychnos colubrina* was shown in figure no.32.

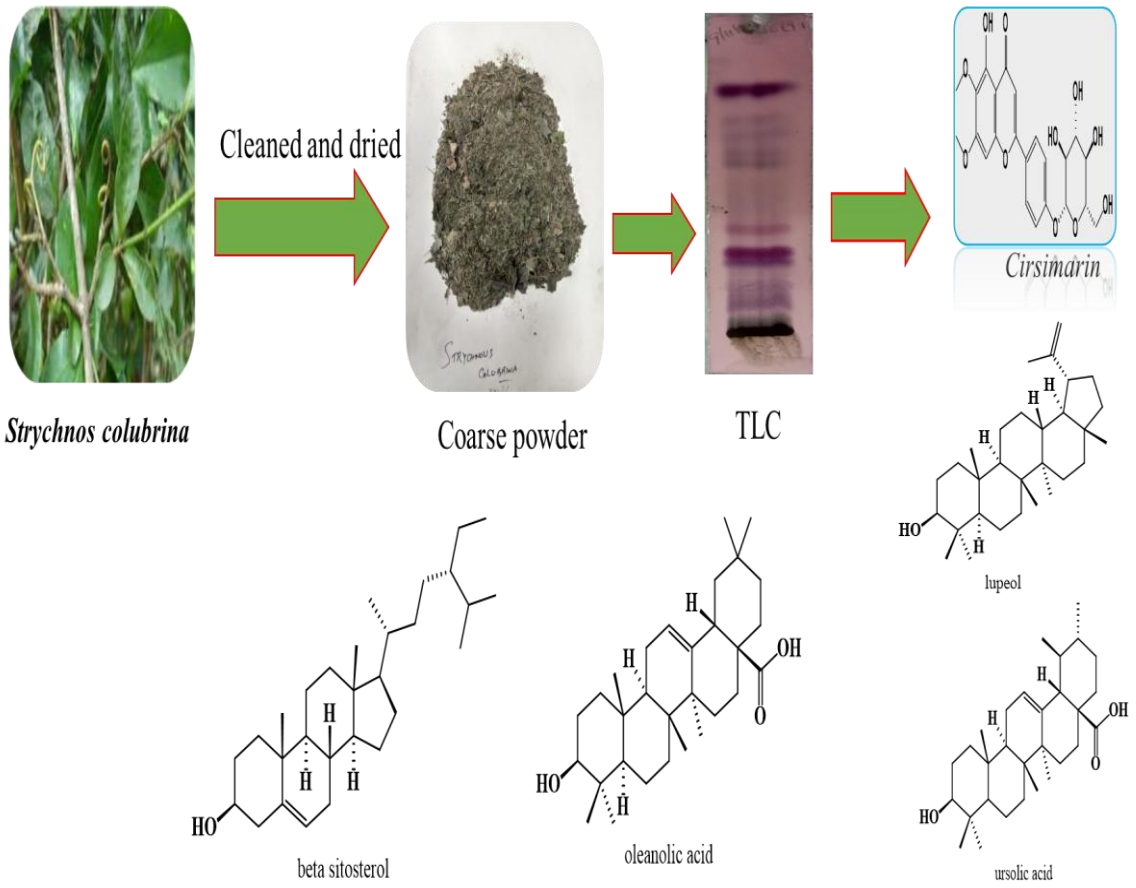

Figure No.32. Isolation of cirsimarin, beta sitosterol, lupeol, Ursolic acid, and olenolic acid from *Strychnos colubrina* plant

5.10 HPTLC, HPLC, Mass spectra and NMR spectra of *Boswellia ovalifoliolata*

5.10.1 HPTLC chromatogram of polarity based different extracts of *Boswellia ovalifoliolata* Sample of different extracts of *Boswellia ovalifoliolata* applied on alumina silica plate and run in the solvent system of hexane : ethyl acetate : formic acid (4.7:5:0.3) and visualized the chromatogram under UV cabinet at 254nm, 366nm and white R.

HPTLC analysis of different extracts of *Boswellia ovalifoliolata* were performed and showed in figure.no. 130. Then calculated the R_f values of the ethanol, hydro alcoholic (0.25, 0.24) at 254nm, chloroform, toluene, ethanol, hydroalcoholic (0.59, 0.6, 0.8, 081) at 366nm and ethanol, hydroalcoholic (0.11, 0.10) at white R.

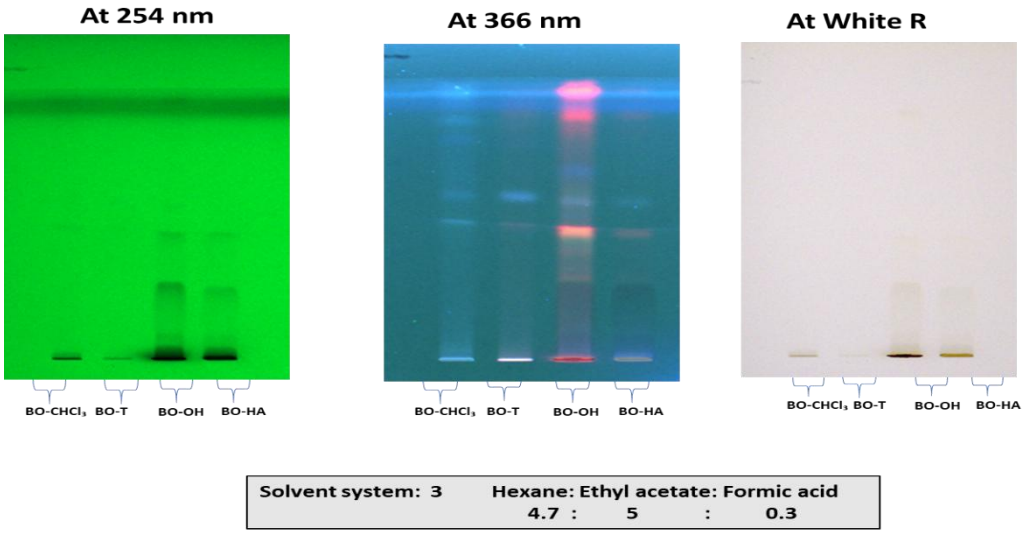

Figure No.33. HPTLC chromatogram of polarity based different extracts of *Boswellia ovalifoliolata*

5.10.2 HPLC chromatogram of Mycertin-7-Glucoside from *Boswellia ovalifoliolata*

The HPLC chromatogram peak of Mycertin-7-Glucoside was eluted at 19.506 min at both 254nm, 366nm with PDA detector and the chromatogram was shown in figure no.34.

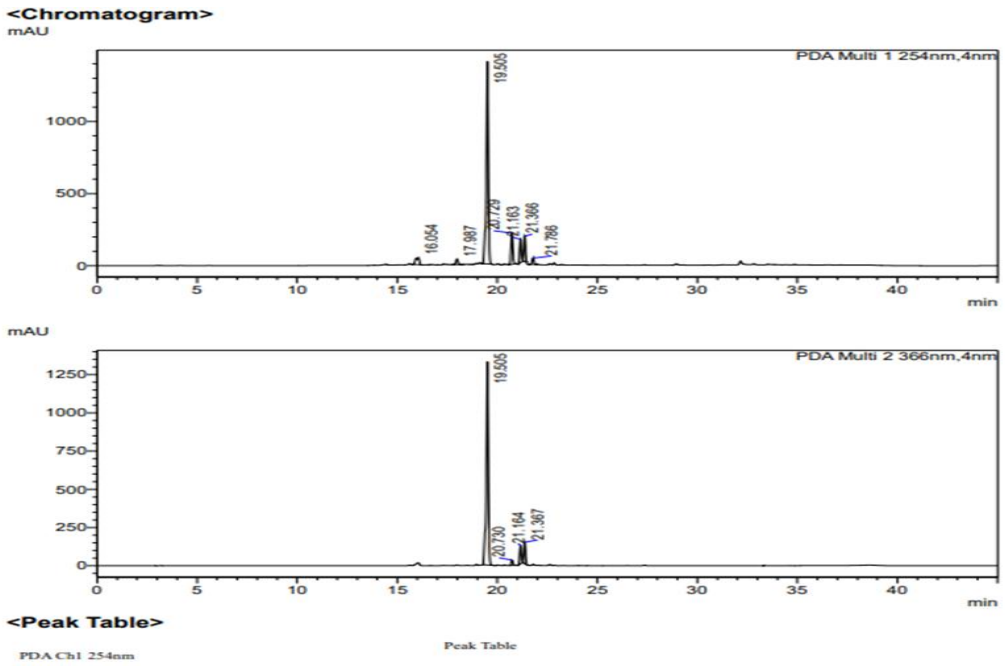

Figure No.34. HPLC chromatogram of Mycertin-7-Glucoside from *Boswellia ovalifoliolata*

5.10.3 Mass spectra of Mycertin-7-Glucoside from *Boswellia ovalifoliolata*

The ES-MS spectrum of Mycertin-7-Glucoside (figure no.132) showed a major peak at m/z (-ev) 480.09 with minor peaks at 349.31. MS/MS analysis on the major ion, m/z (+ev) 480.09.

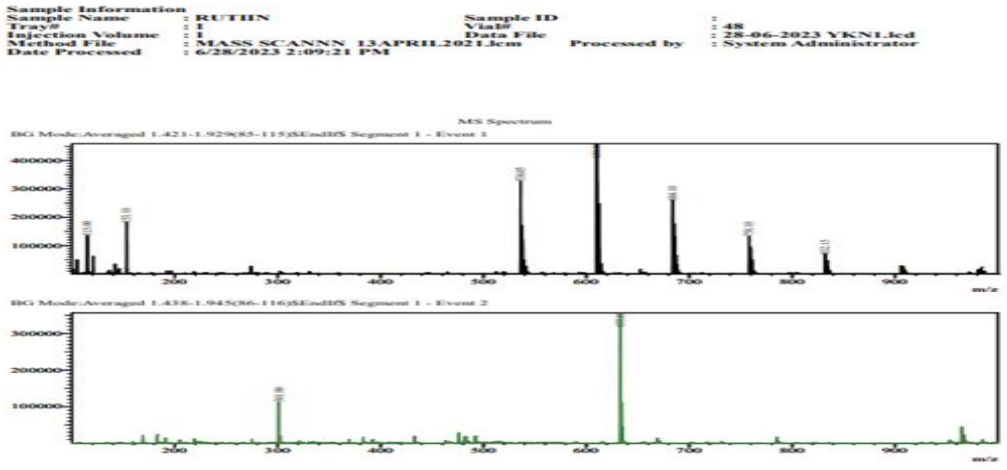

Figure No.35. Mass spectra of Mycertin-7-Glucoside from *Boswellia ovalifoliolata*

5.10.4 NMR spectra of Mycertin-7-Glucoside from *Boswellia ovalifoliolata*

Recorded NMR spectra of Mycertin-7-Glucoside was shown in figure no.36.

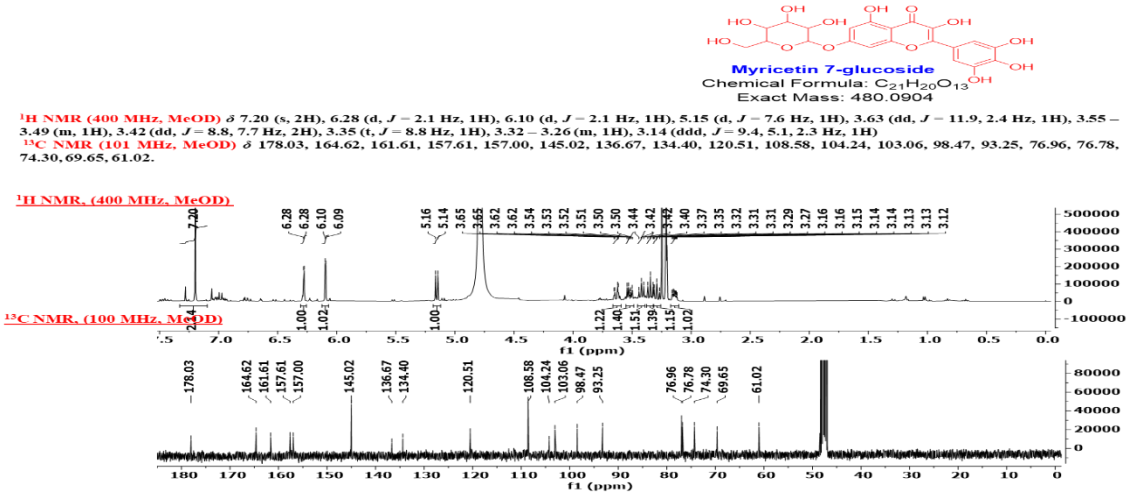

Figure No.36. NMR spectra of Mycertin-7-Glucoside from *Boswellia ovalifoliolata*

5.10.5 ^{1}H and ^{13}C NMR data of Mycertin-7-Glucoside as following:

Mycertin-7-Glucoside: ^{1}H NMR (400 MHz, MeOD) δ 7.20 (s, 2H), 6.28 (d, J= 2.1 Hz, 1H), 6.10 (d, *J*= 2.1 Hz, 1H), 5.15 (d, *J*= 7.6 Hz, 1H), 3.63 (dd, *J*= 11.9, 2.4 Hz, 1H), 3.55-3.49 (m, 1H), 3.42 (dd, *J*= 8.8, 7.7 Hz, 1H), 3.32-3.26 (m, 1H), 3.14 (ddd, *J*= 9.4, 5.1, 2.3 Hz, 1H. ^{13}C NMR (101 MHz, MeOD) δ 178.03, 164.62, 161.61, 157.61, 157.00, 145.02, 136.67, 134.40, 120.51, 108.58, 104.24, 103.06, 98.47, 93.25, 76.96, 76.78, 74.30, 69.65, 61.02 ($C_{21}H_{20}O_{13}$, m/z 480.0904).

5.10.6 Isolation of Myrecitin-7-Glucoside from *Boswellia ovalifoliolata* plant

Isolated molecule of Myrecitin-7-Glucoside from *Boswellia ovalifoliolata* plant was shown in figure no.37.

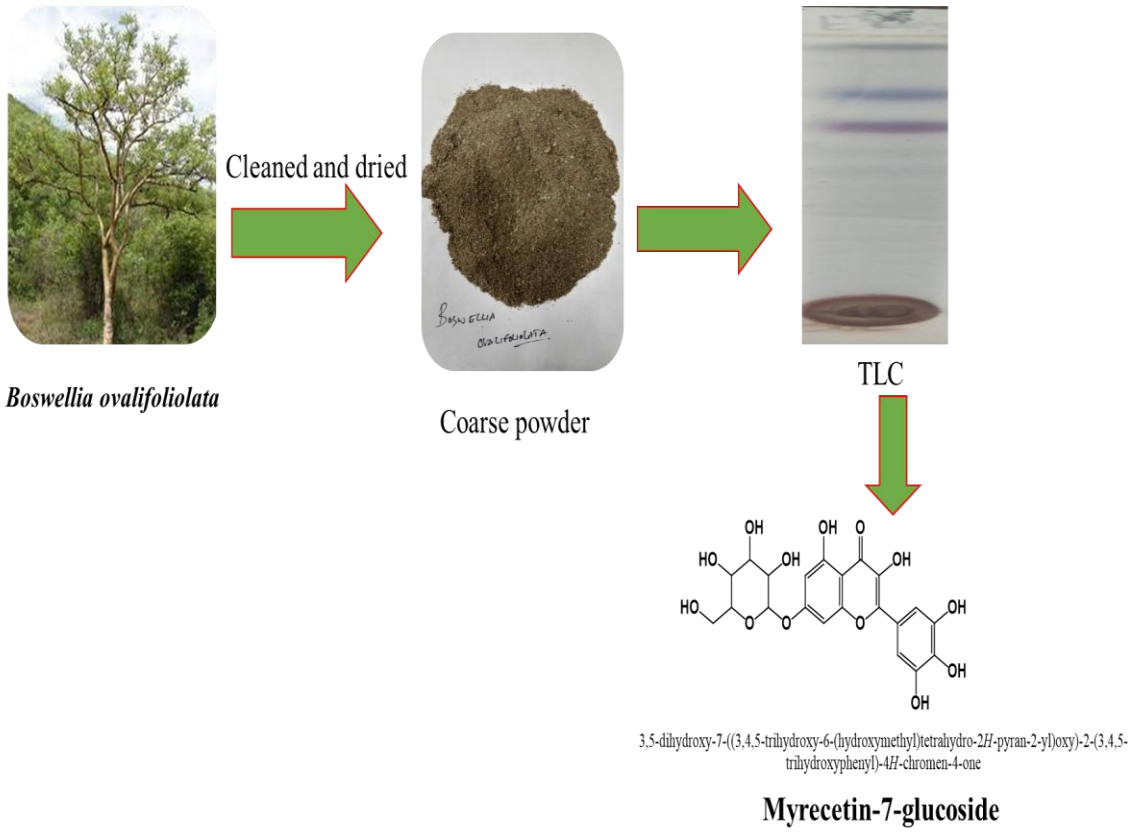

Figure No.37. Isolation of Myrecitin-7-Glucoside from *Boswellia ovalifoliolata* plant

5.11 Screening of various extracts, fractions and isolated compounds for their *in vitro* cytotoxicity

Antiproliferative effect of crude extracts, fractions and isolated compounds was investigated on different human cancer cell lines using SRB assay **(Table no. 18)**. Better antiproliferative efficacy was shown by VL-OH, SC-OH and BO-OH against all cancer cell lines examined with percentage growth inhibition (%GI) at 20 µg/mL values as 75, 70 and 72% against breast (MCF-7), 78, 79, and 91% against colon (HCT-116), 63, 76, and 68% against lung (A549), 23, 34, and 52% against pancreas (MiaPaCa -2), 33, 35, and 36% against glioblastoma (U251) and positive control (5-fluorouracil) shown the %GI as follows 70, 68, 78, 83, and 81% against all the cancer cells.

Cell line type		MCF-7	HCT-116	A549	MIA PaCa-2	U251
Tissue		Breast (20 µg/mL)	Colon (20 µg/mL)	Lung (20 µg/mL)	Pancreatic (20 µg/mL)	Glioblastoma (20 µg/mL)
Plant name	Extracts	% Growth Inhibition				
Vitex leucoxylon	VL-H	59	72	56	39	40
	VL-T	62	73	53	21	42
	VL-$CHCl_3$	60	72	54	21	40
	VL-OH	75	78	63	23	33
	VL-HA	62	75	54	17	30
Strychnos colubrina	SC-H	32	77	53	37	30
	SC-T	39	73	65	48	15
	SC-$CHCl_3$	66	72	65	39	42
	SC-OH	70	79	76	34	35
	SC-HA	67	70	62	36	37
Boswellia ovalifoliolata	BO-H	66	73	63	52	37
	BO-T	47	75	55	67	27
	BO-$CHCl_3$	37	78	59	39	39
	BO-OH	72	91	68	51	36
	BO-HA	57	65	58	76	38

Positive control	Conc. (μM)	Growth Inhibition (%)				
5-fluorouracil	20	70	68	78	83	81

Table No.18. *In-vitro* cytotoxicity of crude extracts of *Vitex leucoxylon*, *Strychnos colubrine* and *Boswellia ovalifoliolata* on human cancer cell line MCF-7, HCT-116, A549, MIA PaCa-2, U251 cell line using SRB assay. The data of anticancer activity is presented as mean observed in three independent experiments.

5.12 Preliminary screening of extracts/fractions against various cancer cell lines of *Vitex leucoxylon*

Antiproliferative effect of crude extracts, fractions and isolated compounds *Vitex leucoxylon* of was investigated on different human cancer cell lines using SRB assay (Table no. 19 & figure no. 20). Better antiproliferative efficacy was shown by VL-OH Extract, VL Fr-10 (Inugalactolipid A), against all cancer cell lines examined with half-maximal inhibitory concentration at (IC_{50}) 2.5, 5, 10, 20, 40 μg/mL values as 33±1.25 against breast (MCF-7), 19±1.16 against colon (HCT-116).

Table. No.19. Preliminary screening of extracts/fractions against various cancer cell lines of *Vitex leucoxylon*

S. No	Cell line type	Conc. (μg/mL)	MCF-7	HCT-116
	Tissue		Breast	Colon
	Extract/fraction		% Growth Inhibition	
1	VL-OH extract	2.5	01	8
		5	14	13
		10	19	25
		20	24	30
		40	46	59
		IC_{50}	>40	33±1.25
2	VL-Fr-2	2.5	-01	21
		5	-10	23
		10	11	24

		20	21	25
		40	53	29
		IC_{50}	38±1.22	>40
3	VL-Fr-3	2.5	11	18
		5	10	-0.2
		10	17	22
		20	22	32
		40	66	41
		IC_{50}	32±1.32	>40
4	VL-Fr-4	2.5	21	13
		5	28	10
		10	50	37
		20	35	66
		40	63	84
		IC_{50}	33±1.05	>40
5	VL-Fr-5	2.5	-2	-5
		5	0.4	35
		10	12	28
		20	41	34
		40	77	38
		IC_{50}	25±1.20	>40
6	VL-Fr-6	2.5	-31	5
		5	-36	7
		10	-28	8
		20	-16	53
		40	5.4	66
		IC_{50}	>40	25±1.28
7	VL-Fr-7	2.5	20	6
		5	26	11
		10	23	8.5

		20	26	27
		40	68	100
		IC_{50}	29±1.17	23±1.23
8	VL-Fr-8	2.5	11	-4
		5	28	-8
		10	45	18
		20	67	0.1
		40	80	99
		IC_{50}	11±1.22	26±1.18
9	VL-Fr-9	2.5	-2	99
		5	-13	99
		10	23	99
		20	46	99
		40	71	99
		IC_{50}	23±1.26	>40
10	VL-Fr-10 **(Inugalactolipid-A)**	2.5	-2	-4
		5	7	-3
		10	30	24
		20	20	54
		40	50	85
		IC_{50}	>40	19±1.16
11	VL-Fr-11	2.5	9	-26
		5	15	-11
		10	15	-15
		20	35	-21
		40	57	-14
		IC_{50}	33±1.27	>40
12	VL-Fr-12	2.5	8	-18
		5	-2	-5
		10	12	-5

		20	25	-2
		40	36	1
		IC_{50}	>40	>40
Positive control	Conc. (µM)	% Growth Inhibition		
5-fluorouracil	20		70	68

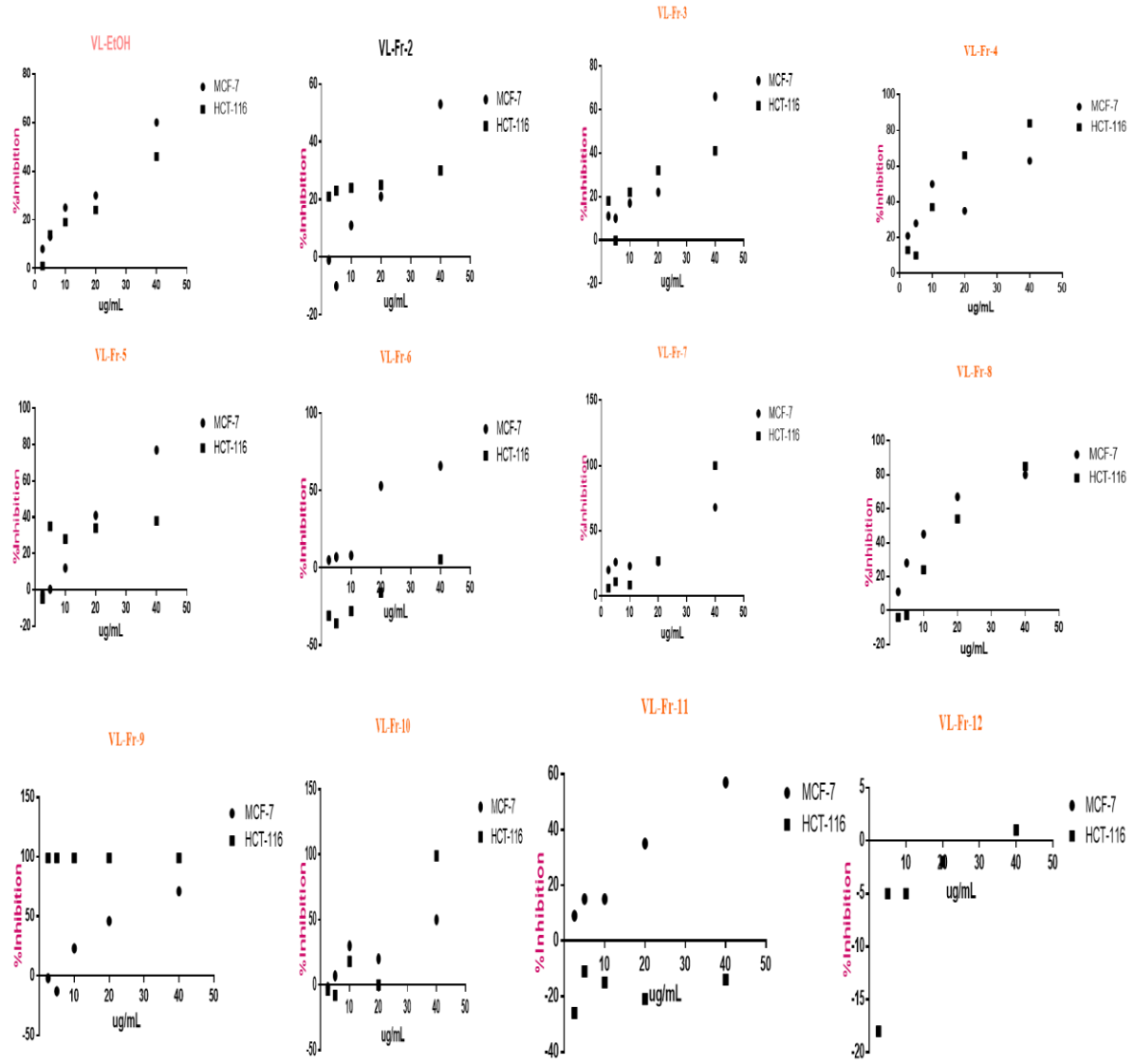

Figure No.20. Showing the IC_{50} graphs of preliminary screening of extracts/fractions (VLOH, VL Fr-2, VL Fr-3, VL Fr-4, VL Fr-4, VL Fr-5, VL Fr-6, VL Fr-7, VL Fr-8, VL Fr-9, VL Fr-10, VL Fr-11, VL Fr-12) against various cancer cell lines (MCF-7 and HCT116) of *Vitex leucoxylon*

5.13 Preliminary screening of extracts/fractions against various cancer cell lines of *Strychnos colubrina*

Antiproliferative effect of crude extracts, fractions and isolated compounds *Strychnos colubrina* of was investigated on different human cancer cell lines using SRB assay (Table no. 20 & figure no. 21). Better antiproliferative efficacy was shown by SC Fr-9 (cirsimarin), against all cancer cell lines examined with half-maximal inhibitory concentration at (IC_{50}) 2.5, 5, 10, 20, 40 µg/mL values as 8±1.21against colon (HCT-116).

Table. No.20. Preliminary screening of extracts/fractions against various cancer cell lines of *Strychnos colubrina*

S. No	Celll line type	Conc. (µg/mL)	MCF-7	HCT-116
	Tissue		Breast	Colon
	Extract/fraction		% Growth Inhibition	
1	SC-OH extract	2.5	01	8
		5	14	13
		10	19	25
		20	24	30
		40	46	59
		IC_{50}	>40	33±1.13
2	SC-Fr-2	2.5	-1	-2
		5	5	-3
		10	17	-5
		20	17	-11
		40	19	20
		IC_{50}	>40	>40
3	SC-Fr-3	2.5	8	3
		5	-2	6
		10	12	9

		20	25	45
		40	36	92
		IC_{50}	>40	23±1.37
4	SC-Fr-4	2.5	30	-0.6
		5	33	6
		10	35	10
		20	45	0.1
		40	49	34
		IC_{50}	>40	>40
5	SC-Fr-5	2.5	0.1	23
		5	22	41
		10	26	74
		20	38	94
		40	52	94
		IC_{50}	>40	15±1.16
6	SC-Fr-6	2.5	-0.8	15
		5	-2	22
		10	-6	51
		20	19	86
		40	25	90
		IC_{50}	>40	9±1.25
7	SC-Fr-7	2.5	16	9
		5	31	10
		10	29	28

		20	38	64
		40	44	90
		IC_{50}	>40	16±1.32
8	SC-Fr-8	2.5	-18	4
		5	-5	12
		10	-5	13
		20	-2	15
		40	1	29
		IC_{50}	>40	>40
9	SC-Fr-9 **(Cirsimarin)**	2.5	16	18
		5	31	35
		10	29	59
		20	38	67
		40	44	75
		IC_{50}	>40	8±1.21
Positive control		Conc (μM)	% Growth Inhibition	
5-fluorouracil		20	70	68

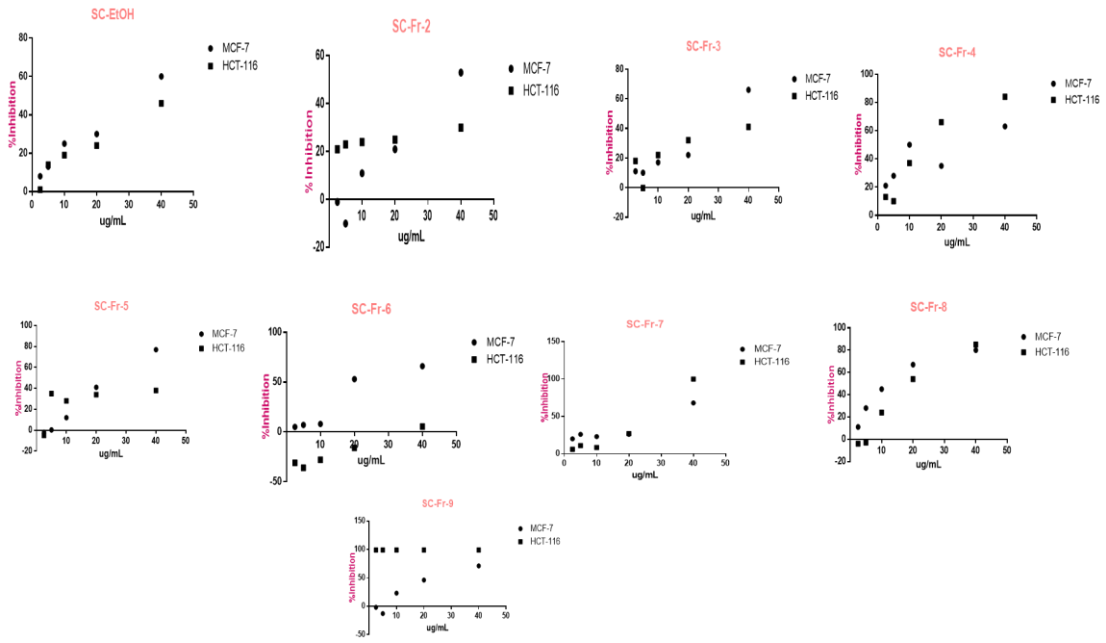

Figure No.21. Showing the IC_{50} graphs of preliminary screening of extracts/fractions (SC-OH, SC Fr-2, SC Fr-3, SC Fr-4, SC Fr-5, SC Fr-6, SC Fr-7, SC Fr-8, SC Fr-9 (Cirsimarin)) against various cancer cell lines (MCF-7, HCT116) of *Strychnos colubrina*

5.14 Preliminary screening of extracts/fractions against various cancer cell lines of *Boswellia ovalifoliolata*

Antiproliferative effect of crude extracts, fractions and isolated compounds was investigated on different human cancer cell lines using SRB assay (Table no. 21). Better antiproliferative efficacy was shown by BO-OH, and BOPPT-12 against all cancer cell lines examined with percentage growth inhibition (%GI) at 20 µg/mL values as 83 and 92% against breast (MCF-7), and 90 and 94% against (HCT116) examined with half-maximal inhibitory concentration at (IC_{50}) 2.5, 5, 10, 20, 40 µg/mL values as 9.14±1.83 (MCF-7) and 5.27±1.19 against colon (HCT-116) cancer cells.

Table. No.21. Preliminary screening of extracts/fractions against various cancer cell lines of *Boswellia ovalifoliolata*

Cell line type		MCF-7	HCT-116
Tissue		Breast (20 µg/mL)	Colon (20 µg/mL)
Plant name	Extract/fraction	% Growth inhibition	
	BO-OH extract	83	92
	BO-PPT-1	42	32

Boswellia ovalifoliolata	BO-PPT-2	56	45
	BO-PPT-3	75	66
	BO-PPT-4	64	53
	BO-PPT-5	37	53
	BO-PPT-6	85	73
	BO-PPT-7	29	54
	BO-PPT-8	42	49
	BO-PPT-9	64	63
	BO-PPT-10	76	74
	BO-PPT-11	39	53
	BO-PPT-12	**90**	**94**
	BO-PPT-13	48	65
	BO-PPT-14	56	48
	BO-PPT-15	59	58
	BO-PPT-16	29	26
	BO-PPT-17	58	48
	BO-PPT-18	74	43
	BO-PPT-19	64	53
	BO-PPT-20	75	73
	BO-PPT-21	41	36
Myricetin-7-Glucoside	**Conc. (µg/mL)**		
	2.5	18	47
	5	31	58
	10	55	67
	20	82	77
	40	92	85
	IC_{50}	9.14±1.83	5.27±1.19
Positive control	Conc (µM)	% Growth Inhibition	
5-fluorouracil	20	70	68

5.15 Mechanism of action of Inugalactolipid-A, Cirsimarin, and Myricetin-7-Glucoside in HCT116 colon cancer cells

5.16 Apoptosis (physiological cell death) in HCT116 colon cancer cell line.

5.17 Inugalactolipid-A, Cirsimarin, and Myricetin-7-Glucoside induces apoptosis of HCT116 cells (DAPI Staining)

Apoptosis induced by the cytotoxic effect of Inugalactolipid-A, Cirsimarin, and Myricetin-7-Glucoside was investigated in HCT116 cells at the concentrations of 2.5, 5, 10, 20μg/mL. Apoptotic changes were revealed by different studies like fluorescence and Bright -field microscopy studies, by using nuclear staining DAPI (4,6 diaminidine-2 phenylindole) dye. Numerous abnormalities or morphological changes like membrane flattening, blebbing, enlarged and uneven pattern in the cell shape with some projections were observed in various Inugalactolipid-A, Cirsimarin, and Myricetin-7-Glucoside treated cells and were compared with untreated cells. DAPI staining in Inugalactolipid-A, Cirsimarin, and Myricetin-7-Glucoside treated cell also revealed that HCT116 cells had died as a result of their nucleus becoming condensed, fragmented, and distorted, as well as the production of apoptotic bodies were observed. In comparison to the control, these alterations were more prevalent in cells treated with Inugalactolipid-A, Cirsimarin, and Myricetin-7-Glucoside and the positive control, Camptothecin. Also, Brightfield microscopic study of HCT116 cells following exposure to Inugalactolipid-A, Cirsimarin, and Myricetin-7-Glucoside for 48 hours showed morphological alterations associated with apoptosis, including roundness, decreased cell quantity and rough surface" (Figure no.22, 23, 24).

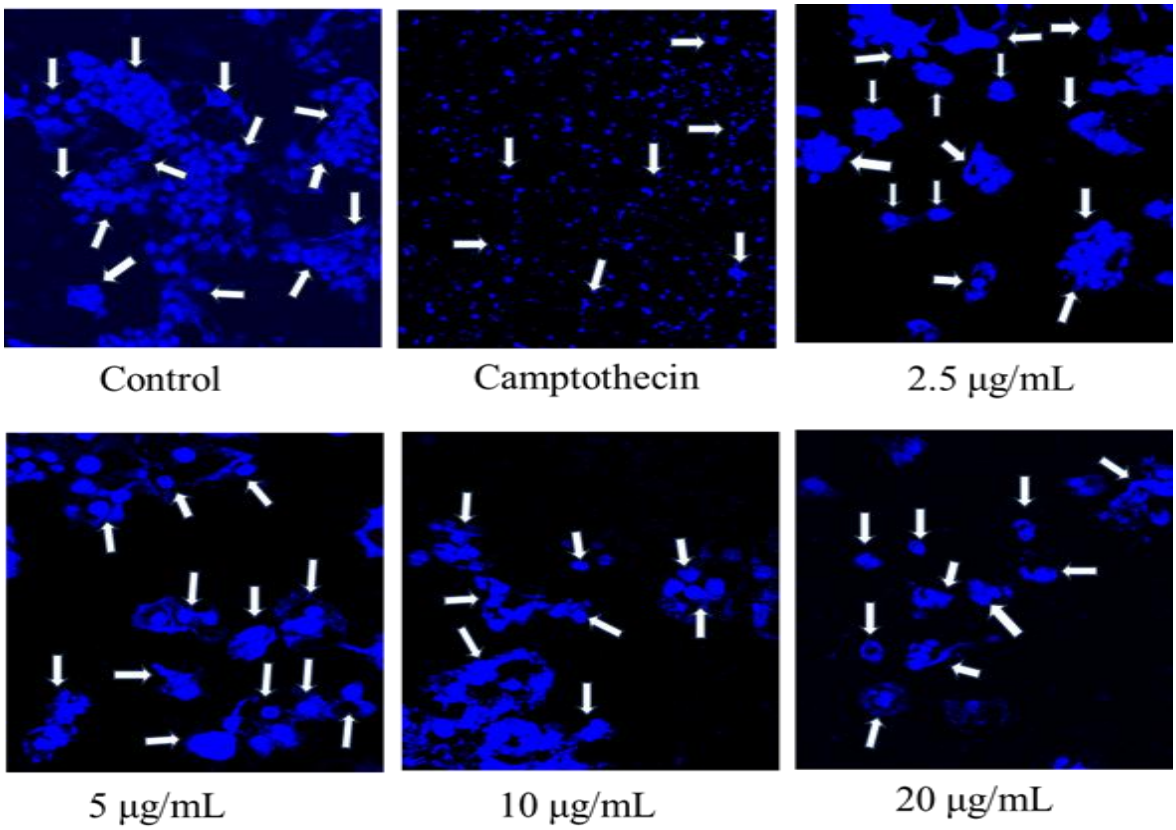

Figure No.22. Inugalactolipid-A induced apoptotic changes in HCT116 cells. Fluorescence microscopic images of DAPI (4,6 diaminidine-2 phenylindole) stained HCT116 cells after Inugalactolipid-A treatment for 48 h showing nuclear morphological changes which include nuclear fragmentation, membrane blebbing and nuclear condensation in concentration dependent manner.

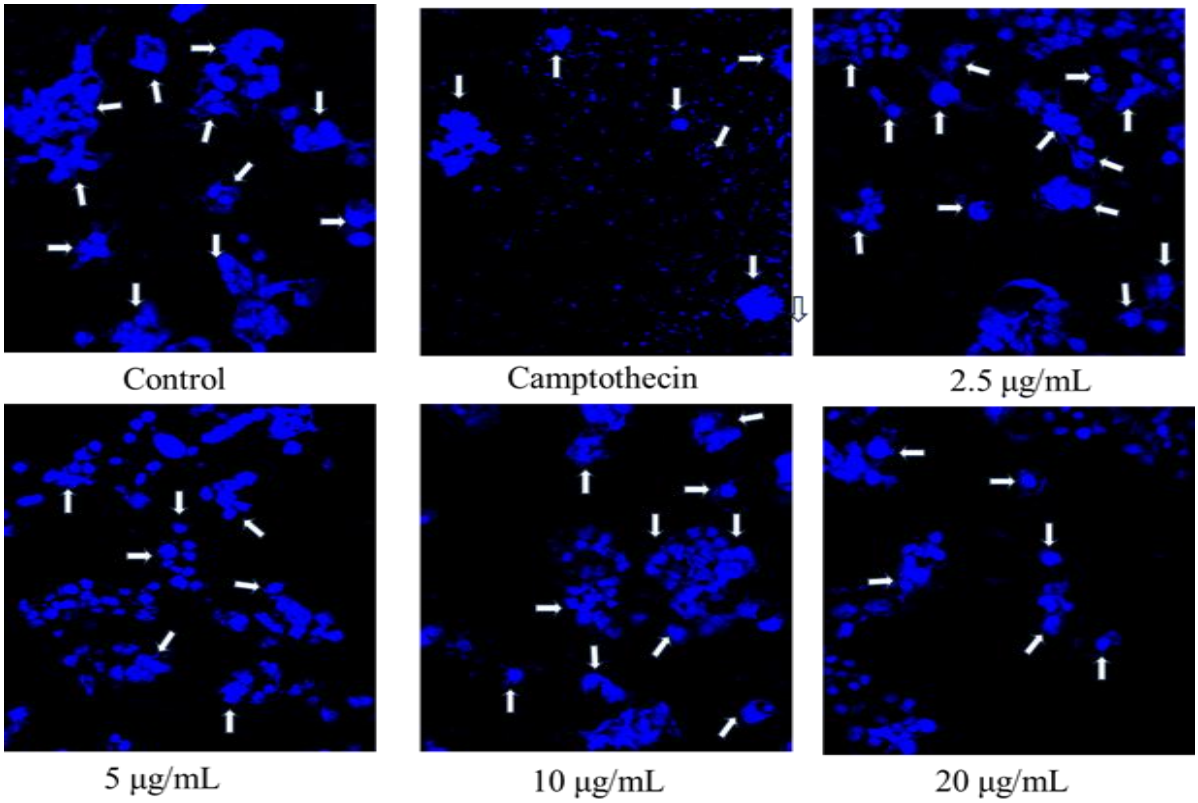

Figure No.23 Cirsimarin induced apoptotic changes in HCT116 cells. Fluorescence microscopic images of DAPI (4,6 diaminidine-2 phenylindole) stained HCT116 cells after Cirsimarin treatment for 48 h showing nuclear morphological changes which include nuclear fragmentation, membrane blebbing and nuclear condensation in concentration dependent manner.

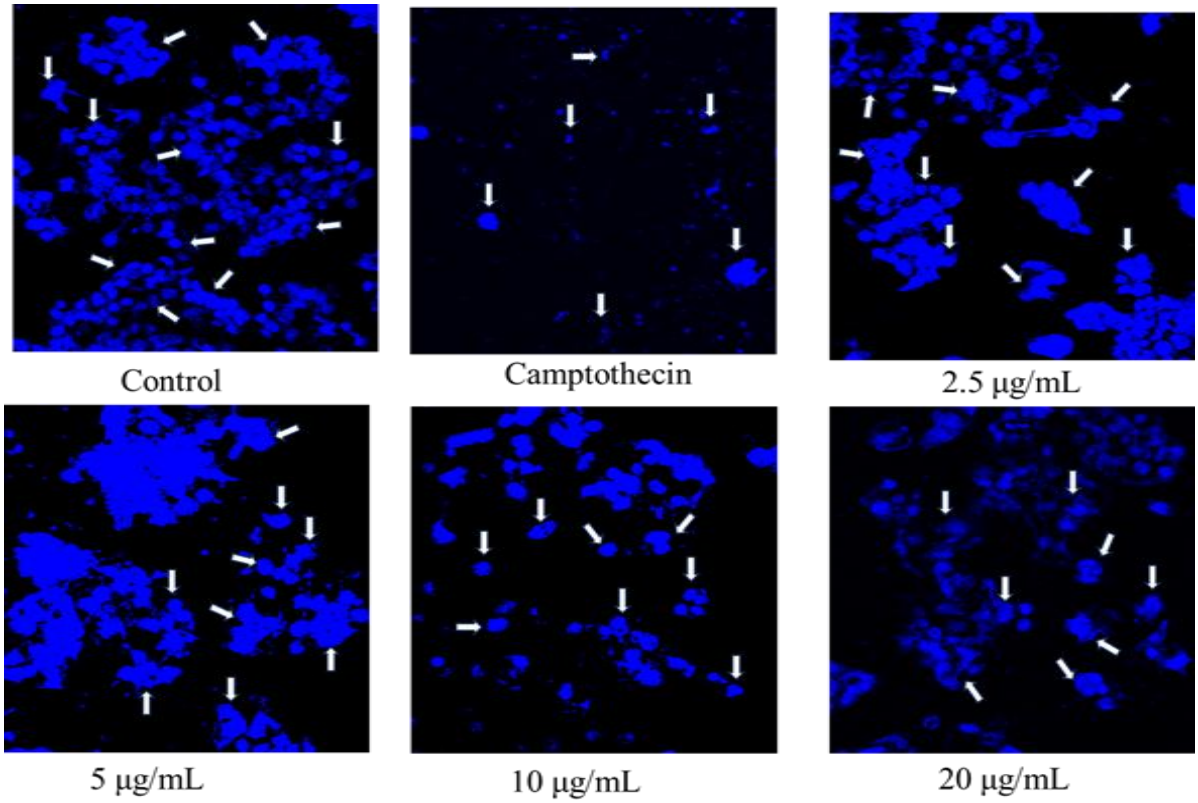

Figure No.24. Myricetin-7-glucoside induced apoptotic changes in HCT116 cells. Fluorescence microscopic images of DAPI (4,6 diaminidine-2 phenylindole) stained HCT116 cells after Myricetin-7-glucoside treatment for 48 h showing nuclear morphological changes which include nuclear fragmentation, membrane blebbing and nuclear condensation in concentration dependent manner.

5.17 Effects of Inugalactolipid-A, Cirsimarin, and Myricetin-7-Glucoside on mitochondrial membrane integrity and release of cytochrome c in HCT116 colon cancer cells.

Inugalactolipid-A, Cirsimarin, and Myricetin-7-Glucoside induced mitochondrial membrane integrity and other mitochondrial dysfunction was determined by treating HCT116 cells at different doses (2.5, 5, 10, and 20µg/mL) for 48 h with Inugalactolipid-A, Cirsimarin, and Myricetin-7-Glucoside. Using fluorescence microscope cells stained with Rh-123 were observed. Figure no.137, 138 and 139, showed that the control had the highest fluorescence intensity (100 %). Inugalactolipid-A (97%, 73%, 15% and 7.5%), Cirsimarin (70%, 61%, 10.5% and 6.21%), and Myricetin-7-Glucoside (74%, 62%, 11% and 6.85%) treated cells exhibited decreasing fluorescence intensity at 2.5, 5, 10, 20µg/mL respectively which is statistically significant ($P<0.05$) when compared to control. This demonstrate that there occurs depolarization of mitochondria in HCT116 cells which was apparently induced by

Inugalactolipid-A, Cirsimarin, and Myricetin-7-Glucoside resulting in a substantial loss of MMP (Δ Ψ m) with increase in the concentration of Inugalactolipid-A, Cirsimarin, and Myricetin-7-Glucoside. Similarly, the MMP of HCT116 cells treated with camptothecin (positive control) was turn down to 10% of the control (Figure 25, 26, 27).

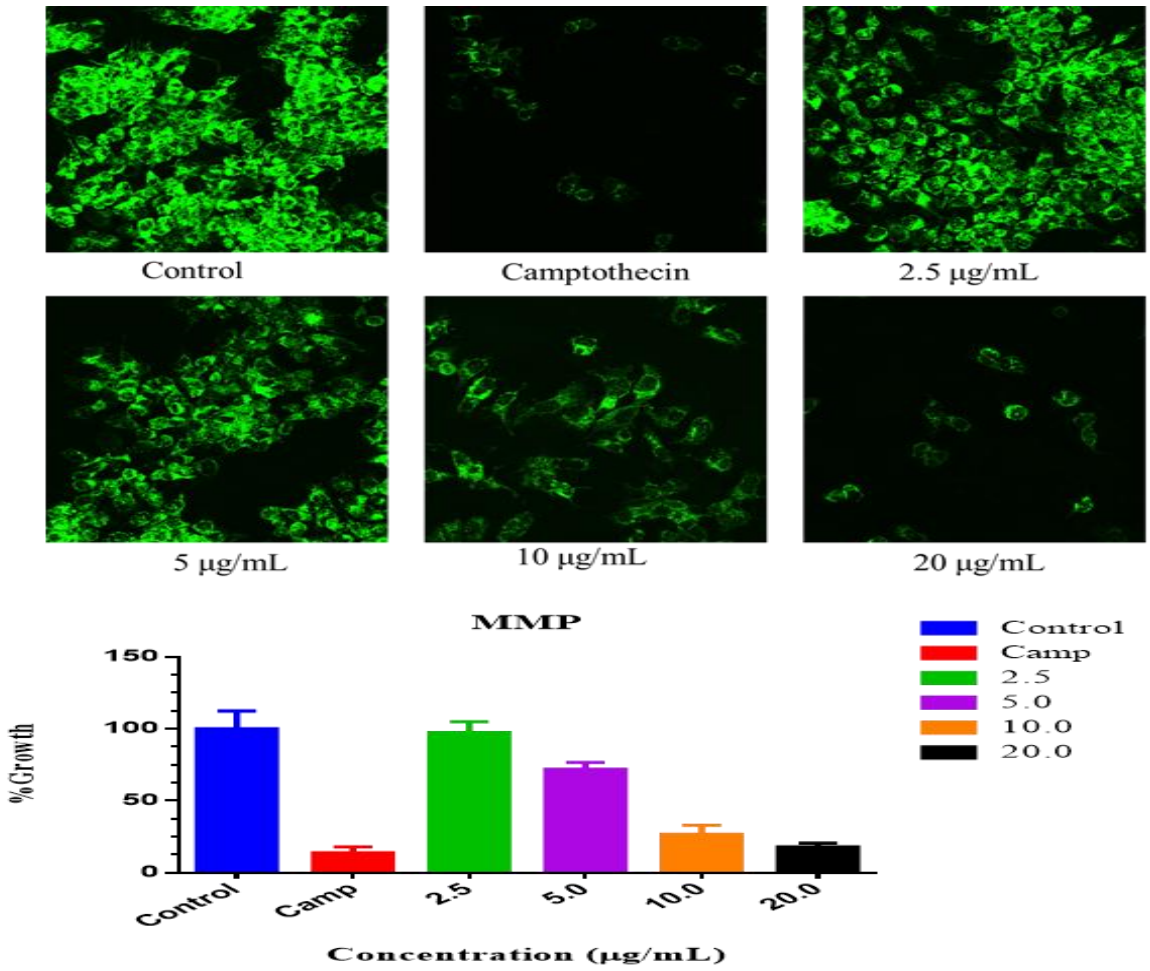

Figure No.25. Inugalactolipid A induced mitochondrial membrane depolarization in HCT116 cells. Cells were stained with Rhodamine 123. MMP loss was assessed using fluorescence microscopy upon treatment with Inugalactolipid A at concentrations of 2.5, 5, 10, 20µg/mL. The fluorescence intensity decreased with the increase in the concentration. Camptothecin was used as a positive control.

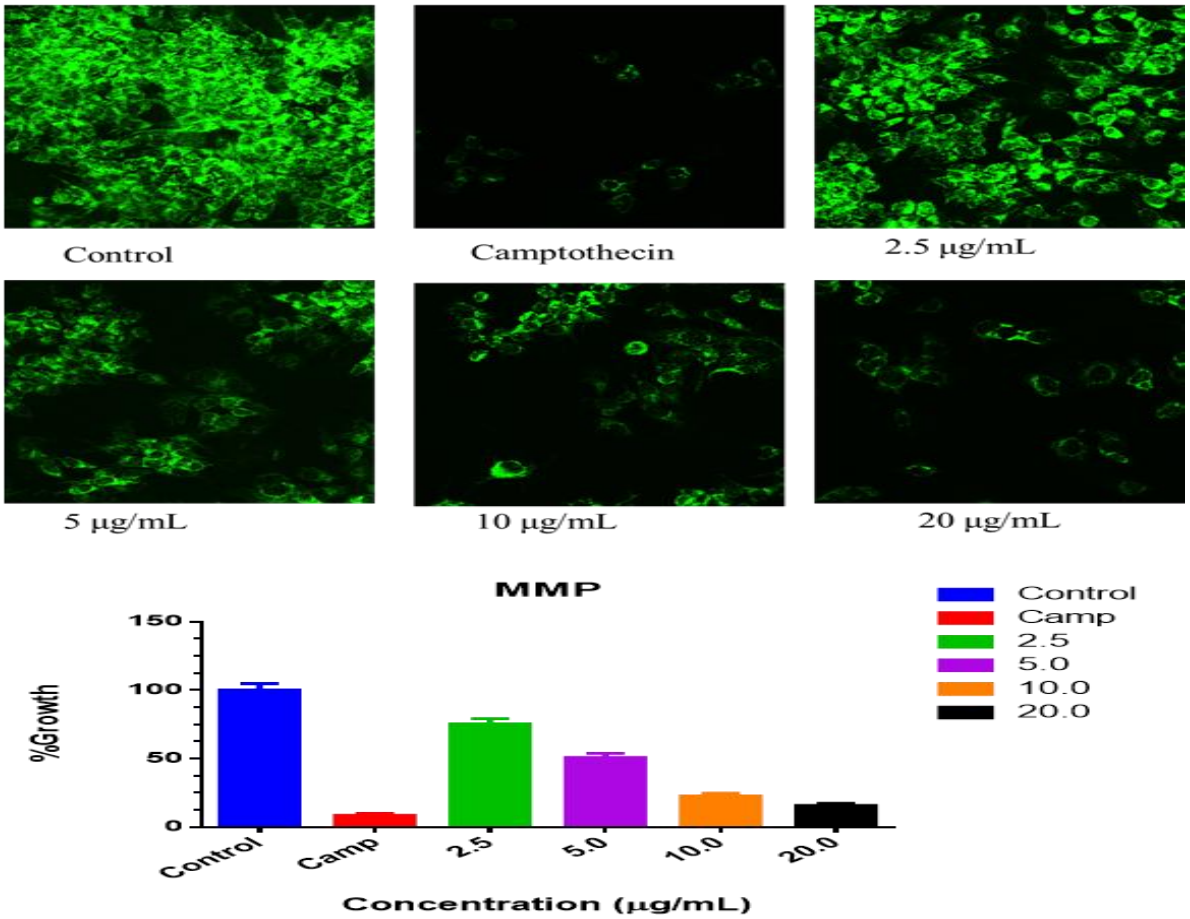

Figure No.26. Cirsimarin induced mitochondrial membrane depolarization in HCT116 cells. Cells were stained with Rhodamine 123. MMP loss was assessed using fluorescence microscopy upon treatment with Cirsimarin at concentrations of 2.5, 5, 10, 20µg/mL. The fluorescence intensity decreased with the increase in the concentration. Camptothecin was used as a positive control.

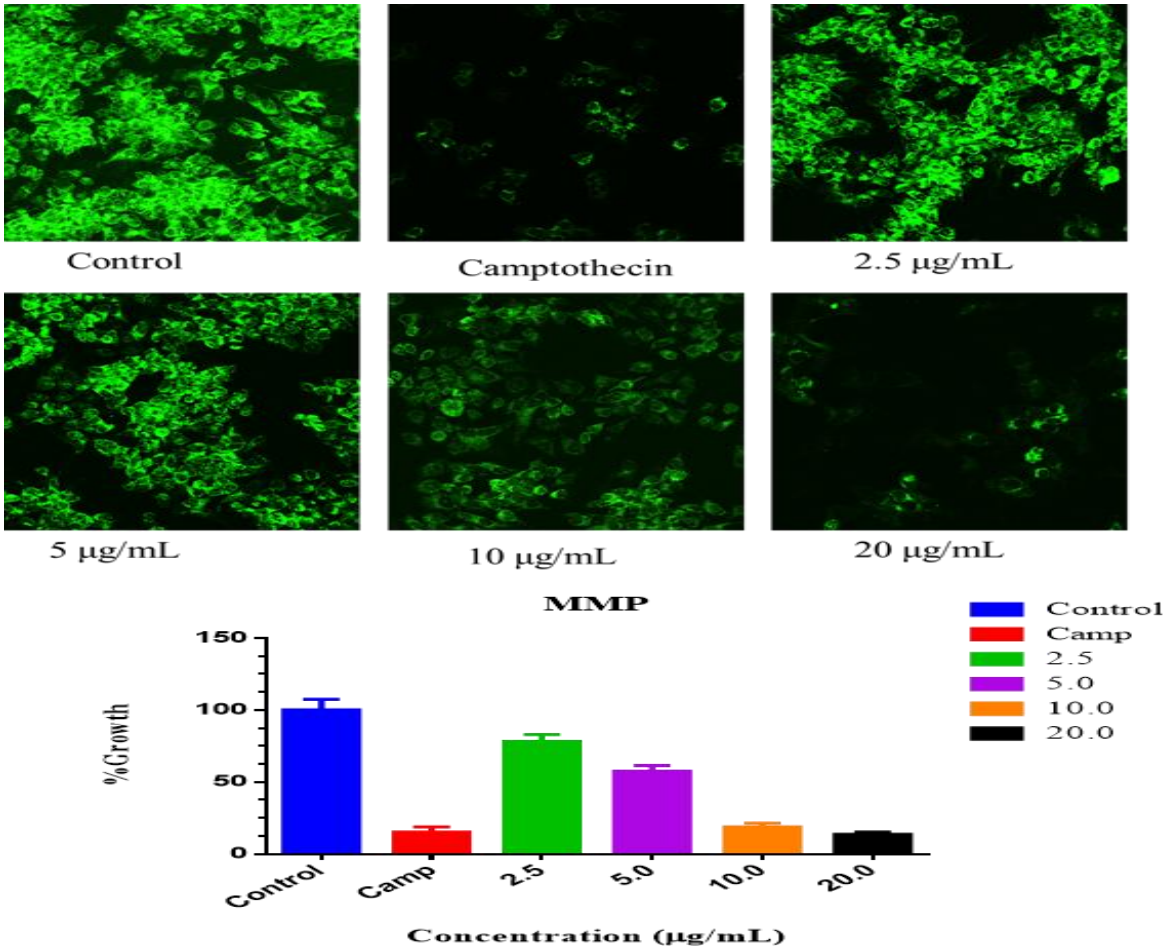

Figure No.27. Myricetin 7-glucoside induced mitochondrial membrane depolarization in HCT116 cells. Cells were stained with Rhodamine 123. MMP loss was assessed using fluorescence microscopy upon treatment with Inugalactolipid A at concentrations of 2.5, 5, 10, 20µg/mL. The fluorescence intensity decreased with the increase in the concentration. Camptothecin was used as a positive control.

5.18 Generation of ROS in HCT116 colon cancer cells

Mitochondria are the major sites of reactive oxygen species (ROS) production in mammalian cells, and the loss of MMP commonly results in ROS generation. Therefore, we investigated whether MMP loss imposed by Inugalactolipid A, Cirsimarin, and Myricetin-7-glucoside influenced ROS generation in HCT116 cells. With the help of DCFH -DA fluorescent probe for ROS, we measured the ROS levels in HCT116 cells. Fluorescence microscopy analysis showed 11%, 45%, 58%, and 75% elevation of ROS at 2.5, 5, 10, and 20µg/mL concentrations of Inugalactolipid A, Fluorescence microscopy analysis showed 9%, 18%, 52%, and 77% elevation of ROS at 2.5, 5, 10, and 20µg/mL concentrations of Cirsimarin, Fluorescence microscopy analysis showed 21%, 55%, 78%, and 85% elevation of ROS at 2.5, 5, 10, and 20µ concentrations of Myricetin-7-Glucoside, respectively as compared to the control sample

(Figure no. 28, 29, 30).

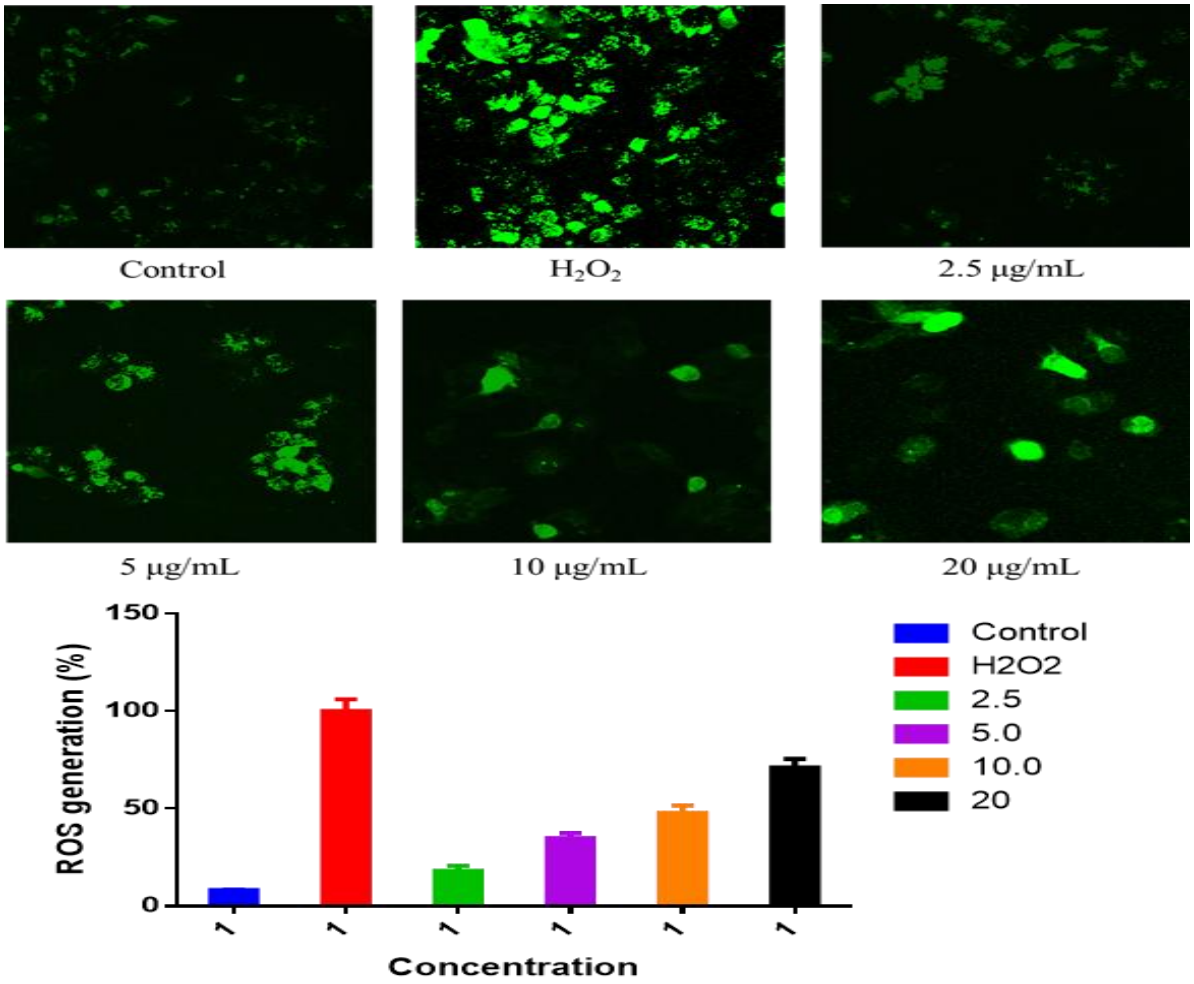

Figure No.28. Treatment with Inugalactolipid-A resulting in the production of reactive oxygen species (ROS). HCT116 cells were treated with different concentrations of Inugalactolipid-A for 48 h for ROS generation. Figure represents the fluorescent intensity of DCFH-DA dye through fluorescent microscopy. The fluorescence intensity increased with the increase in the concentration. H_2O_2 was used as a positive control.

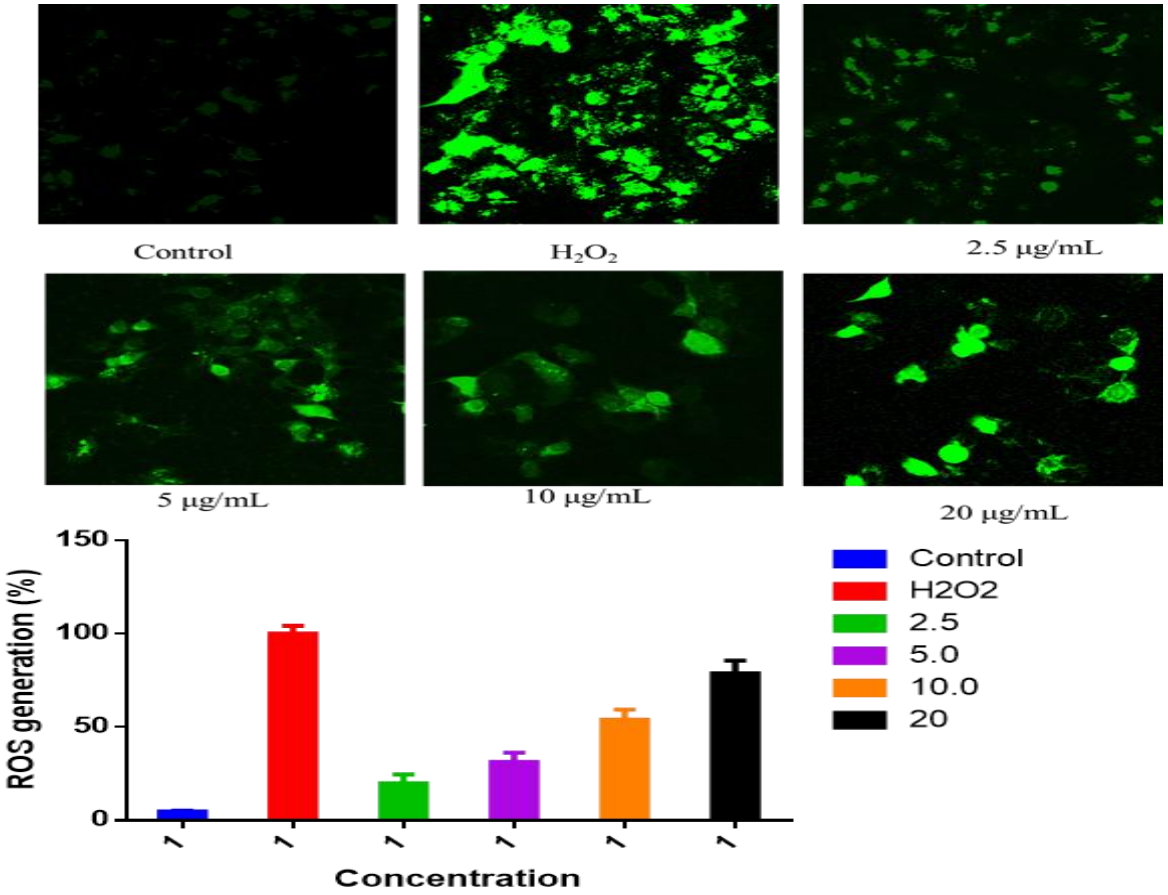

Figure No.29. Treatment with Cirsimarin resulting in the production of reactive oxygen species (ROS). HCT116 cells were treated with different concentrations of Cirsimarin for 48 h for ROS generation. Figure represents the fluorescent intensity of DCFH-DA dye through fluorescent microscopy. The fluorescence intensity increased with the increase in the concentration. H_2O_2 was used as a positive control.

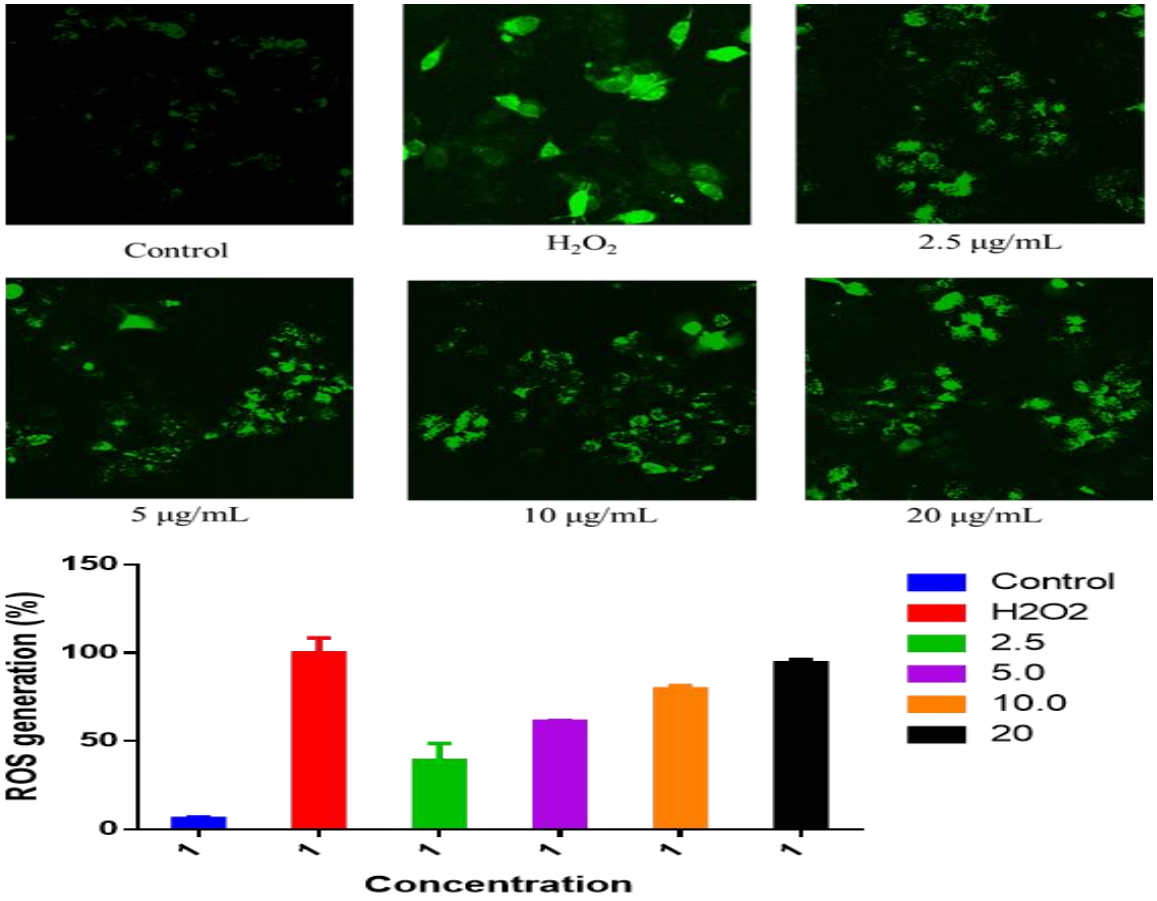

Figure No.30. Treatment with Myricetin-7-glucoside resulting in the production of reactive oxygen species (ROS). HCT116 cells were treated with different concentrations of HCT116 for 48 h for ROS generation. Figure represents the fluorescent intensity of DCFH-DA dye through fluorescent microscopy. The fluorescence intensity increased with the increase in the concentration. H_2O_2 was used as a positive control.

6. SUMMARY AND CONCLUSION

Summary

Plants have an ancient legacy of being used in cancer treatment. A study has reported nearly 3000 plant species that have anti-cancer properties. The development of numerous anticancer medications currently being used in clinical settings is due to plant-based pharmaceuticals. Vinca alkaloids, epipodophyllotoxin lignans, taxane, diterpenoids, steroidal saponins, and derivatives of camptothecin quinoline alkaloid make up the majority of plant-based anticancer drugs The dissertation entitled "Phytochemical Investigation and Anticancer Potential of the Selected Medicinal Plants of Seshachalam hills, Andhra Pradesh", representing the work on leaves of *Vitex leucoxylon*, *Strychnos colubrina*, and *Boswellia ovalifoliolata* were found mostly in Seshachalam hills, Andhra Pradesh. The plant material was collected from the Seshachalam hill ranges of Tirupati District, Andhra Pradesh, India. Selected medicinal plants were unexplored medicinal herbs commonly used by indigenous people in the management of various health complications in Tirupati, Chittoor district of Andhra Pradesh. Phytochemical screening suggests that ethanolic extract of *Vitex leucoxylon*, *Strychnos colubrina*, and *Boswellia ovalifoliolata* contains valuable phytoconstituents like alkaloids, flavonoids, phenols, terpenoids and steroids.

Medicinal plants play an important role in the basic healthcare systems of countries. The therapeutic properties of the plant are determined by the biological substances present in the plant components that are used to produce medicinal remedies. *Vitex leucoxylon*, *Strychnos colubrina* and *Boswellia ovalifoliolata* belongs to the Lamiaceae, Loganiaceae and Burseraceae family. These medicinal plants were widely distributed in the Eastern and Western Ghats of India, as well as in Sri Lanka. Traditionally, the leaves of this plant have been extensively used to treat leprosy, cancer, as an emetic, and for headaches. The phytochemical screening identified the presence of alkaloids, flavonoids, tannins, phenols, steroids, glycosides, and anthraquinone. Phytochemical investigations were confirmed by chromatographic techniques (TLC, HPTLC, GC-MS and HPLC) and isolated compounds (Inugalactolipid A, Cirsimarin, and Myricetin-7-Glucoside) were confirmed by spectroscopic methods (IR, MS, HR-MS, and NMR). Inugalactolipid A, Cirsimarin, and Myricetin-7-Glucoside that was isolated from the leaves of *Vitex leucoxylon*, *Strychnos colubrina*, and *Boswellia ovalifoliolata*, exhibited excellent cytotoxicity against the (MCF-7 and HCT116) cell lines when tested against a panel of cancer cell lines. These two cell lines were further selected for mechanistic evaluation of Inugalactolipid A, Cirsimarin, and Myricetin-7-

Glucoside. Various *in vitro* assays such as DAPI staining, MMP and ROS were performed to understand the cell death by Inugalactolipid A, Cirsimarin, and Myricetin-7-Glucoside.

DAPI staining and ultrastructural observations through SEM revealed multiple abnormalities and morphological changes like membrane blebbing, enlargement, flattening and irregularity in the shape of cells with some projections in Inugalactolipid A, Cirsimarin, and Myricetin-7-Glucoside treated cell as compared to untreated cells.

Inugalactolipid A, Cirsimarin, and Myricetin-7-Glucoside caused loss in mitochondrial membrane integrity that led to a significant loss of MMP. Reactive oxygen species (ROS) are mostly generated in mitochondria of human cells, and ROS are frequently produced when MMP breaks down. In HCT116, Inugalactolipid A, Cirsimarin, and Myricetin-7-Glucoside caused MMP loss thereby affecting intracellular ROS level.

In conclusion, our findings show that isolated compounds of Inugalactolipid A, Cirsimarin, and Myricetin-7-Glucoside inhibits the growth of HCT116 cells by inducing DNA damage, mitochondrial membrane potential and elevating intra-cellular ROS level.

These experimental results suggest that the of *Vitex leucoxylon*, *Strychnos colubrina*, and *Boswellia ovalifoliolata* possess significant phytochemicals and anticancer potentials.

Future Scope

The present study involved phytochemical investigation and anticancer potentials of the Seshachalam hills, Tirupati, Andhra Pradesh available species of *Vitex leucoxylon*, *Strychnos colubrina* and *Boswellia ovalifoliolata* in three different cancer cell lines to establish the evidence of their anticancer potential. Presence of various secondary metabolites in the plants were also investigated with the help of TLC, HPTLC, GC/MS and HPLC. However, lot of work could be done in following areas:

- Isolation and characterization of the secondary metabolites responsible for the anticancer effects.
- With the isolation of secondary metabolite, the possible mechanism of action of anticancer effects can be investigated.
- The plants could be developed as an adjuvant therapy to the available anticancer medicines.

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